Verticillium dahliae var. longisporum Stark. attacking oilseed rape (Brassica napus L. subsp. oleifera) and the myrosinase/glucosinolate system in compatible/incompatible interactions.

Karapapa, Vassiliki

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by
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A thesis submitted for the degree of
Doctor of Philosophy

King's College
University of London

November 1995

Gratefully dedicated
στον
αγαπημένο μου σύντροφο και φίλο
Γιωργάκη

Ἀλλὰ τὰ πρῶτα εἰ μέλλει δηλώματά τινων γίγνεσθαι, ἔχεις τινὰ καλλίω τρόπον τοῦ δηλώματα αὐτὰ γενέσθαι ἄλλον ἢ αὐτὰ ποιῆσαι ὅτι μάλιστα τοιαῦτα οἷα ἐκεῖνα ἃ δεῖ δηλοῦν αὐτά;
ΠΛΑΤΩΝ
Κρατυλος, 433d

(But if the primary names are to be representations of any things, can you suggest any better way of making them representations than by making them as much as possible like the things which they are to represent?)

Abstract

Verticillium wilt in winter-sown oilseed rape (*Brassica napus* L. subsp. *oleifera*) cvs was shown to be caused by the host-specific stable diploid strain viz. *Verticillium dahliae* var.*longisporum* Stark.

Thirty-nine isolates were obtained from different hosts, 23 from oilseed rape, 5 from other Brassicas and related species, as well as 9 isolates of *V.dahliae* and 2 of *V.albo-atrum* from non-Cruciferous hosts, and various locations (Germany, Sweden, Japan, France, Poland, Greece, Spain, UK, Italy and USA). These isolates were investigated to establish the distinct nature of the host-specific strain viz. *V.d.longisporum.* This strain was distinguished from haploid strains of *V.dahliae* by a combination of morphological characters (type of microsclerotium, conidial length, number of phialides/node in conidiophores), enzyme tests (extracellular polyphenol oxidase activity), diploidy levels (DAPI fluorescence staining and Feulgen DNA microdensitometry of conidial nuclei), DNA-fingerprinting (Random amplified polymorphic DNA, [RAPD], using 3 different primers), chromosome-length polymorphisms by Pulsed Field Gel Electrophoresis [PFGE], and green-house pathogenicity tests employing 5 different cvs of winter oilseed rape and a root-dip inoculation method. On the above basis, a new species name is proposed here for this host-specific pathotype viz. *Verticillium cruciferarum* sp. nov.

Localization of the enzyme myrosinase was performed *in situ* by immunogold labelling (using two polyclonal antibodies). In the radicle, myrosinase was found in myrosin grains in specific cells (myrosin cells) in the outer-most layer of the cortex. In 4-6 week-old plants, the enzyme also occurred in vesicles in sub-epidermal cortical parenchyma cells of the root tip. Myrosin cells were also found in the phloem of the stem and petiole at this time. In stems and petioles of plants inoculated with the virulent isolate 161, myrosinase was localized **both** in phloem cells **and** in xylem contact parenchyma cells. However, in control healthy stems and petioles, and those of symptomless plants inoculated with avirulent isolate 130, the enzyme was restricted to the phloem only.

The specific activity of the host plant enzyme myrosinase was investigated in a compatible interaction using isolate 161 of the pathogenic diploid strain viz. *V.d.longisporum* and in an incompatible interaction using isolate 130 of a typical haploid strain (tomato) of *V.dahliae.* There was an increase in specific activity of myrosinase in extracts of oilseed rape (cv. Cobra) hypocotyls at 10 days after inoculation with the avirulent isolate 130, whereas a decrease in such activity was observed for '161-infected' hypocotyls at that time. Levels of individual glucosinolates (gluconasturtiin [GLNS] and gluconapin [GLNP]) by 15 days post-inoculation were increased in roots and hypocotyls in an incompatible interaction, whereas they decreased in a compatible interaction. On the contrary, at 10-15 days post-inoculation, the levels of these individual glucosinolates in leaves and cotyledons of '161-infected' plants were higher as compared with those of control plants and those inoculated with the avirulent isolate 130.

These results are discussed in relation to recognition and suppressor models of host-pathogen interactions.

Acknowledgements

I should like to sincerely thank my supervisor Dr. J.B. Heale for his supervision throughout this project and critical reading of the thesis. His continual help in terms of scientific advises and moral support are greatly appreciated.

Valuable discussions with Dr. B. Bainbridge (King's College) , Dr. J. Rossiter and Dr. D. James (Wye College), Prof. A. Bones (Unigen, University of Trondheim), Dr. K. Zeise (University of Rostock) and their co-workers during collaborative work are gratefully acknowledged.

My thanks are also extended to numerous colleagues and friends at King's College who have helped me both in and out of the lab, particularly my friend Jovita Madeira and the EM staff at Wye College Mr. I. Brown and Ms S. Reardon for their technical support.

Thanks are also due to my relatives and friends outside college who morally supported me during the work; my mother Αγγελα, my auntie Παρασκευη, my young brother Γιαννη, Αμαλια, Νασσο, κ. Θαναση, κ. Αθηνα, and my friends Nick, Αρη, Katerina, Margarita, Margaret, Χριστινα and Βουλα.

Finally, I would like to thank my supervisor in Greece, Prof. E. Tjamos, who initiated me for the path of study on the plant pathogenic fungus *Verticillium dahliae.*

The deepest thanks to Γωργακη for all his love, support, encouragement, advice and patience. This thesis is as his as it is mine.

This work was partially supported by a postgraduate research studentship from the Institute of Greek State Scholarships.

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Aims of the project

A. To establish the distinct nature of the host-specific strain of *Verticillium dahliae*, i.e. *V.dahliae* var. *longisporum*, that causes vascular wilt disease in oilseed rape and related species by investigating (1) the ploidy levels in ungerminated conidia (by Feulgen DNA microdensitometry and DAPI-stained nuclear diameters), (2) their associated morphological characters (conidial length measurements, observations on microsclerotia formation and morphology of conidiophores, in a number of culture media), (3) their enzymatic characters (extracellular, polyphenol oxidase, and polygalacturonase, activity), and by (4) DNA-fingerprinting (Random Amplified Polymorphic DNA [RAPDs] and Chromosome-Length Polymorphic DNA, [PFGE]) in different isolates from *Brassica* and non-*Brassica* hosts. All morphological, enzymatical and genetic characters were investigated in association with the virulence of the different isolates to oilseed rape cultivars.

B. To elucidate the resistance mechanism of oilseed rape cultivars (cvs) of *Brassica napus* to isolates of *Verticillium dahliae* var. *longisporum* by investigating the rôle of glucosinolates and myrosinase during host-parasite interactions involving isolates of pathogenic and non-pathogenic strains of the fungus: More specifically to determine the cellular and sub-cellular localization and activation of the host enzyme myrosinase (immunogold localization of the enzyme using polyclonal antibodies and *in vitro* measurements of the activity of the enzyme) and the post-infection level of total and individual glucosinolates (HPLC analysis) in relation to the localization of the fungus within the host tissues.

1. Introduction

1.1. The crop

Oilseed rape, *Brassica napus* subsp. *oleifera* L. belongs to the family Brassicaceae (Cruciferae) in the order Capparales. The word 'rape' originates from the Latin name *rapum*, meaning turnip. On the contrary to other oilseeds, rapeseed does not come from one species. In Asia there are three related, but different, species: *Brassica napus* (rape), *B.campestris* (=*B.rapa*, turnip rape) and *B.juncea* (leaf mustard); in Europe and America there are two species: *B.napus* and *B.campestris* that are also subdivided into subsp. The subsp. *oleifera* is predominately used for vegetable oil. There is also winter rape (var. *biennis*) and spring rape (var. *annua*) that differ with respect to cultivation techniques, crop yield and chemical composition. For this investigation, 5 different cvs of winter oilseed rape (*Brassica napus* subsp. *oleifera* var. *biennis*) were used: Cobra, Envol, Idol, Samourai and Falcon.

Brassica napus is an amphidiploid species (n=19, genome aacc). The botanical relationships of common rapeseed species have been shown by U (1935). The *B.napus* (n=19, genome aacc) was derived (see Figure -1.1- below) by hybridization of the two different basic diploid species *B.oleracea* (n=9, genome cc) and *B.rapa* (n=10, genome aa).

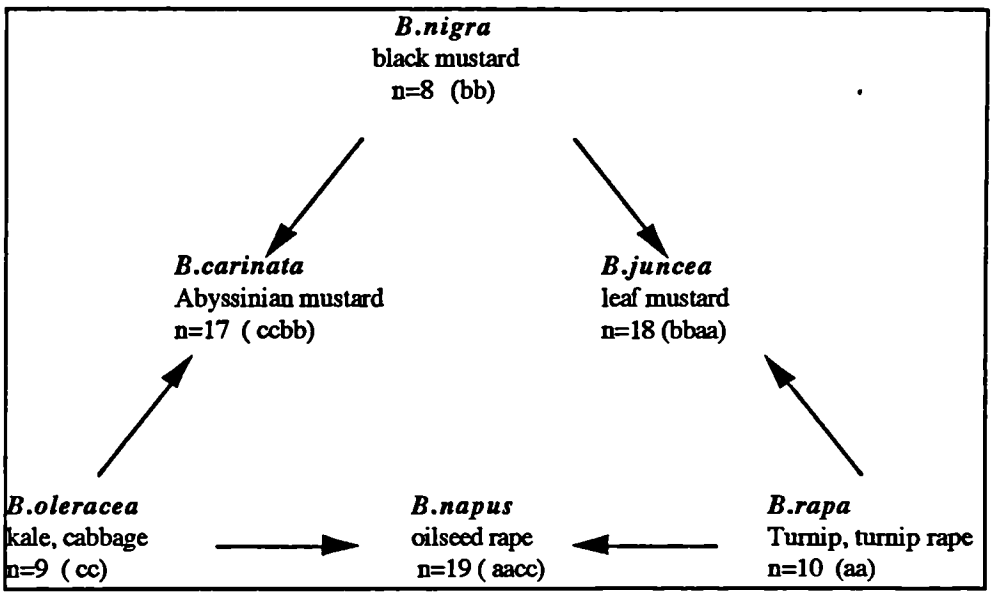


Figure -1.1- Botanical relationship of important Brassica species according to U (1935).

Oilseed rape is cultivated widely in China, Canada and India; within the EU, France and UK are the main producers. Rapeseed is the only oil-producing plant species of economical importance capable of successful cultivation in the northern latitudes of Europe. The primary economic reason for cultivation of rapeseed is as a source of human oil consumption. Its oil content ranges from 40-60% (Godin & Spensley, 1971), depending on the varieties used. It is employed in the manufacture of salad dressings, table oils and confectionery fats and shortening, which are used in the making of cakes, biscuits, pastries and many other

products. It is also used in industry (tanning), for lubricating of machines, in soaps and detergents, and recently as a diesel fuel; thus it has the potential to become a very important, non-food, EU crop.

Erucic acid is one of the numerous fatty acids present in rape oil, and it exists in a far higher concentration in this oil than any other vegetable oil. The traditional varieties of rapeseed contained 22-60% erucic acid in their oils, producing high erucic acid oil (HEAR) (Shahidi, 1990). When rape oil was fed to laboratory animals in a series of tests during the 1960s, an abnormal number of heart lesions developed and this was attributed to erucic acid. No such effects have been recorded in humans (Niewiadonsky, 1990). Nevertheless, it was decided that the erucic acid content of rape seed should be reduced and plant breeders developed varieties possessing reduced levels of erucic acid. The low erucic acid (LEAR) oil containing 5% or less erucic acid was first produced in Canada in 1968. Low erucic acid is now defined as a variety in which erucic acid constitutes less than 2% of the measured fatty acid in the seed. Such a variety is referred as a 'single low' variety.

The rapeseed meal or cake which remains, when the oil has been extracted from the seed, is used for animal feeding (ruminants, pigs and poultry). Rapeseed is a good source of high quality protein (30-40%). The proteins in meal have a valuable amino acid composition (Ohlson & Anjou, 1979), with a higher content of essential amino acids (lysine, methionine, cysteine, threonine and tryptophan), as compared with other cereals and legumes (Larsen & Sörensen, 1985). The factor mostly limiting the utilization of rapeseed meal as a protein supplement is the presence of glucosinolates, and the variety of undesirable products formed by their cleavage. Certain glucosinolates with a (i) ß-hydroxylated alkenyl side chain (napoleiferin, progoitrin) or a (ii) indolyl side chain, convert into goitrogenic products, such as 5-vinyloxazolidine-2-thione (VOT) and the thiocyanate anion respectively. These compounds cause thyroid enlargement by interfering with the synthesis of thyroxin in the case of VOT or compete with iodine for uptake by the thyroid gland in the case of thiocyanate ion and adversely affect growth and reproduction (Fenwick *et al.*, 1983). Furthermore, volatile isothiocyanates produced by the cleavage of gluconapin and glucobrassicanapin reduce palatability of the meal. Other breakdown products of glucosinolates (i.e. nitriles) are toxic, and can cause significant abnormalities to body organs in animals with these products in their diets. The majority of the breakdown products are volatile and extremely pungent. Therefore, with the discovery of the Polish variety 'Bronowski' in 1968 as a genetic donor of low glucosinolate content, plant breeders immediately started programmes for the development of 'double zero' varieties with reduced levels of both erucic acid and glucosinolates in the seed. 'Bronowski' contains tenfold lower levels than do the original rapeseed varieties. The name 'canola' was adopted in 1979 in Canada for all 'double-low' (00) cultivars. The reduction in glucosinolates has been exclusively due to a reduction in alkenyl glucosinolates. The indole glucosinolates which occur in minor, but significant amounts, remained constant. By the most recent definition, 'canola' refers to a rapeseed cultivar that contains less than 2% erucic acid in its oil, and less than 30 µmol/g of one or any of the four known aliphatic glucosinolates (viz. gluconapin, progoitrin, glucobrassicanapin and gluconapoleiferin) in its air-dried defatted meal (Adolphe, 1980). The specification for the 'canola' standards does not refer to the indole glucosinolates or the other glucosinolates that are present in the meal in smaller and variable amounts. The recent 'double low' varieties have 10-20µmoles/g glucosinolates.

The first oilseed rape 'double-low' variety called 'Erglu' was a spring variety licensed in Europe in 1973. 'Librador' was the first 'double-low' winter variety licensed by the German authority in 1981 (Röbbelen & Brauer, 1990).

Oilseed rape is now cultivated in every county in the UK were arable agriculture is practised; it is one of the three (including barley, wheat) most important arable crops. The major proportion of the crop is winter-sown (83%) due to its higher yield and the earlier harvest (from July to early August), as compared with spring-sown varieties, giving plenty of time for cleaning preparations when winter wheat is to follow (NIAB, 1993). In the UK, the 'double low' character in seed is measured in all varieties grown by the National Institute of Agricultural Botany (NIAB) or the Agricultural Development and Advisory Service (ADAS) in variety trials. Both indole and alkenyl glucosinolates are reported. The designated limits of the Commission of the European Community (CEC) are 35 μmol/g of seed at 9% moisture content.

Although the level of rapeseed glucosinolates is under genetic control, a number of environmental factors (soil composition, application, weather) have been shown to influence seed glucosinolate concentration (Milford & Evans 1991), and to lead to levels of glucosinolates that exceed the proposed limits. There is a considerable variation in seed glucosinolate content between sites and years for a single variety, but the effecting factors have not yet been elucidated (Askew, 1990). The application of sulphate can increase the glucosinolate content, while nitrate may reduce its levels; stress factors such as drought and plant density can also increase glucosinolates levels (Heaney & Fenwick, 1987). Zhao *et al.* (1994) showed the influence of N and S applications on the seed glucosinolate profile of winter oilseed rape. A high N supply increased the relative proportion of progoitrin (2-hydroxybut-3-enyl) glucosinolate at the expense of glucobrassicanapin (pent-4-enyl) in the seeds of the double low variety Cobra. In addition S deficiency depressed the biosynthesis of alkenyl glucosinolates more than that of the indole.

The main reasons that make oilseed rape so popular in Britain is the ease with which the crop can be grown, its suitability to a wide range of soil types, its tolerance to variation in climate, its usefulness as a cleaning crop and as a good preparation for winter wheat. Above all though, the popularity of oilseed rape is definitely due to its profitability as compared with that of most other break crops in the UK (Ward *et al.*, 1985).

1.2. The genus *Verticillium*

1.2.1. Taxonomy of *Verticillium dahliae* and *V.albo-atrum* based on morphological characters

The Genus *Verticillium* is a member of the Hyphomycetes, Deuteromycotina (Fungi Imperfecti) which are known to occur only in the vegetative mycelial form and conidial form of the true fungi: Eumycota. The Genus was established in 1816 by Nees Von Esenbeck based upon its characteristic verticillate conidiophores bearing whorls of phialides. It reproduces asexually (anamorph) via the production of single-celled, uninucleate asexual spores i.e.: conidia produced from the tip of each phialide, forming conidial heads in a mucilaginous secretion.

Species identification has been made mainly using morphological characters.

The five most important plant-pathogenic fungi in the Genus are *Verticillium dahliae* Kleban, *Verticillium albo-atrum* Reinke and Berthold, *Verticillium nigrescens* Pethybridge, *Verticillium nubilum* Pethybridge, and *Verticillium tricorpus* Isaac which are clearly distinct from each other by the different formation of resting structures (Isaac 1949, 1953). No perfect state has been found for any of these 5 species.

Three species have been described as causing vascular wilt disease, *Verticillium dahliae* Kleb. (1913), *Verticillium albo-atrum* Reinke and Berthold (1879) and *V.tricorpus* Isaac (1953). They are separated taxonomically by morphological characters; the formation of true microsclerotia; dark resting mycelium, and both types of resting structure plus chlamydospores respectively.

Reinke and Berthold (1879) first described *V.albo-atrum* as an imperfect pathogen which formed resting mycelium, torulose black mycelia which were responsible for the black or dark brown discoloration of the infected potato tissue. They called the resting mycelium 'Dauermycelien' or 'Sclerotin' or 'Zellhaufen'.

Verticillium dahliae, first isolated from dahlia by Klebahn (1913), was described as forming microsclerotia which arose from irregular, multicellular septation and budding of the cells of a single hypha, and not from the anastomosis of mycelium. He considered this to be distinct from the resting mycelium of *Verticillium albo-atrum* and concluded that this fungus isolated from dahlia should be considered as a different, distinct species i.e. *V.dahliae* Kleb.

Isaac (1949), in a comparative study of the morphology, physiology and pathogenicity of certain *Verticillium* isolates, concluded that three distinct organisms or groups could be distinguished. Type M (microsclerotial formation) assigned as *Verticillium dahliae* Klebahn formed microsclerotia arising from the budding of a cell in all planes followed by dark pigmentation. Type D (dark mycelium) assigned as *Verticillium albo-atrum*, formed dark resting mycelium arising from the septation and darkening of hyphae. Type C (chlamydospores) assigned as *Verticillium nigrescens* Pethybridge formed small chlamydospores. He could also distinguish them by physiological and pathogenicity characters. Isolates of group M grew moderately well at 30^{0}C and some growth was still observed at 32.5 ^{0}C whereas, isolates of type D produced yeast-like cultures at 32.5 ^{0}C and failed to grow at 35 ^{0}C. M type isolates produced wilt

symptoms in inoculated hosts up to 27 ^{0}C, whereas isolates of type D produced wilt only up to 21.5 ^{0}C. Hastie (1978) later showed that interspecific diploids from complementary auxotrophs from *V.dahliae*, *V.albo-atrum*, and *V.tricorpus* rarely formed new genotypes, indicating that these species comprised non-homologous genomes.

Verticillium albo-atrum (see Figure-1.2-) is distinguished according to C.M.I. description (Hawksworth & Talboys, 1970) as follows: Colonies are white-greyish, flocculose and whitish-cream in reverse. Conidiophores abundant, more or less erect, hyaline, verticillately branched, 2-4 phialides arising at each node, phialides sometimes secondarily branched; conidiophores characteristically **darkened at the base** when growing on plant tissue. Conidia arise singly at the apices of the phialides, ellipsoidal to irregularly sub-cylindrical, hyaline, mainly simple, occasionally 1-septate, 3.5-10.5 (-12.5) X 2-4µm. After 2-3 weeks, cultures become brownish-black due to the formation of dark resting mycelium, which forms by septation and swelling of hyphae that appear torulose in parts, **never budding to form microsclerotia-like structures**. The resting mycelium walls darken due to an non-indolic pigment, allomelanin, Gafoor & Heale (1971a,1971b). Production of dark resting mycelium tends to be 'lost' after prolonged subculturing in the laboratory. Chlamydospores are apparently only produced in some isolates of *V.albo-atrum* from alfalfa (Harris, 1985) and microsclerotia are totally absent. Often, white raised sectors appear on colonies grown in Petri dish cultures. The fungus survives as dark resting mycelium up to 9-10 months in the soil under the conditions used by Heale & Isaac (1963).

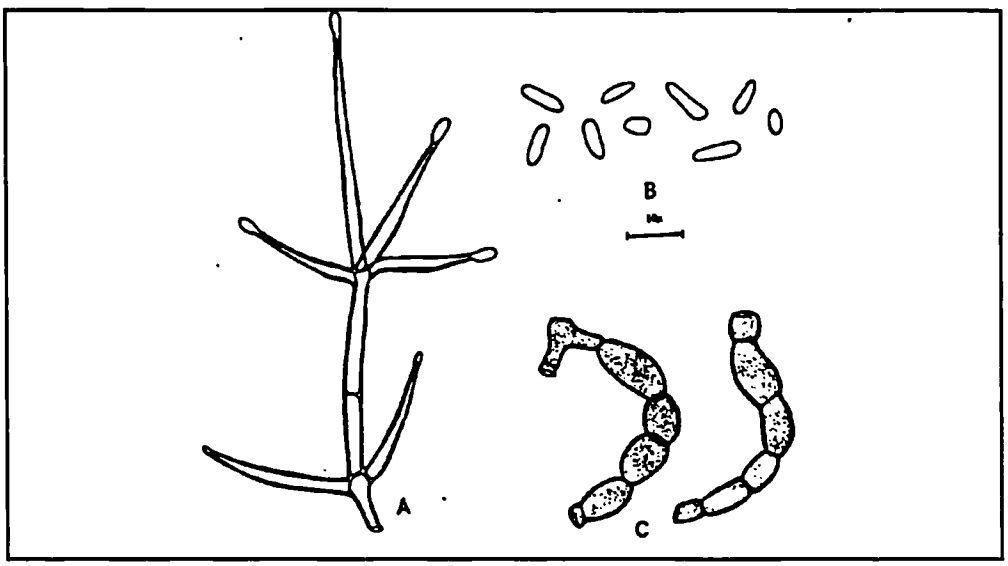


Figure-1.2- *Verticillium albo-atrum* Reinke & Berthold (1879). A: Verticillate conidiophore; B: conidia; C: dark resting mycelium. (From C.M.I. Descriptions of Pathogenic Fungi and Bacteria No. 255, Hawkworth & Talboys, 1970).

Verticillium dahliae (Figure-1.3-) also forms rather similar conidiophores, more or less erect, hyaline, verticillated branched with 3-4 phialides arising at each node, phialides secondary branched. However, its conidiophore never has the darkened base as that of *V. albo-atrum*. Phialides variable in size,

mainly 16-35 X 1-2.5 µm. Conidia arise singly at the apices of the phialides, ellipsoidal to irregularly sub-cylindrical, hyaline, mainly simple but occasionally 1-septate, 2.5-8 X 1.4-3.2 µm. Microsclerotia are the only resting structures formed; limited developing of dark brown resting mycelium is only formed directly in association with microsclerotia and chlamydospores are absent. Microsclerotia formed in culture are dark brown to black, torulose or botryoidal, consisting of swollen, almost globular cells.

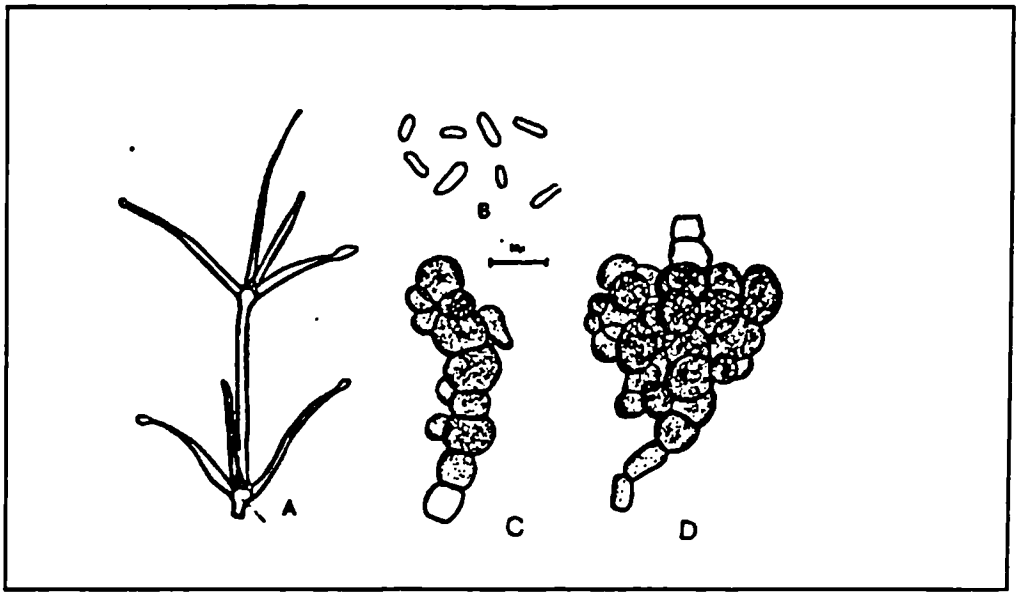


Figure-1.3- *Verticillium dahliae* Klebahn (1913). A: Verticillate conidiophore; B: conidia; C: young microsclerotium; D: mature microsclerotium. (Based on C.M.I. Descriptions of Pathogenic Fungi & Bacteria No. 256, Hawkworth & Talboys, 1970).

Each microsclerotium develops initially by enlargement of individual hyphal cells of a single hypha or adjacent hyphae. From this cluster or chain of nearly globose cells, the microsclerotium increases in size by **repeated budding** as well as from the larger budded cells (Brown & Wyllie, 1970). These authors were actually using the *V.dahliae* microsclerotial form, but misnamed their isolates as *V.albo-atrum*. Budding continues throughout the development of the microsclerotium, resulting in a compact mass of chains of globose cells. As the microsclerotium matures, melanin accumulates as granules in the microsclerotia cell walls and in a fibrillar network encapsulating the walls (Wheeler *et al.*, 1976). The same authors, using albino and brown microsclerotia mutants showed that 3,4-dihydro-3,6,8 trihydroxy-1 (2H) naphthalenone [(+)-scytalone]-treated albino mutants formed melanin granules and turned black as in the wild-type, suggesting that scytalone is a natural intermediate in melanin synthesis. Bell *et al.* (1976), using UV-induced mutants of the T9 defoliating strain of *V.dahliae* from cotton, showed also that (+)-scytalone was the intermediate in melanin biosynthesis which involved at least two or more enzyme steps; a dehydratase and an alcohol dehydrogenase and the intermediate compounds 1,3,8-trihydroxynaphthalene(1.3.8. T.H.N.), 3,4 dihydro 3,8, dihydroxy-1 (2H)-naphthalenone (vermelone) and 1,8-dihydroxynaphthalene, which by further oxidation and polymerisation, forms a non-indolic allomelanin. Melanin is important for protection of cell walls against the action of microbial chitinase and

endo-ß-1,3 glucanase (Bloomfield & Alexander, 1967) and also protects fungal cells from injury caused by irradiation and desiccation (Durrell, 1964; Zhdanova & Pokhodenko, 1973). Microsclerotia are very variable in shape, elongate to irregularly spherical, very variable in size, 15-50 (-100) µm diameter. White sectors that do not produce microsclerotia commonly appear in cultures. Microsclerotia can survive in soil for at least 14 years (Domsch *et al.*, 1980). Typas & Heale (1978) showed that the development of darkly pigmented resting structures is controlled by a number of non-allelic genes, some of which are cytoplasmic (Hyl) and some are nuclear in origin. There was evidence for the irregular segregation of the cytoplasmic Hyl marker, as compared with nuclear auxotrophic markers.

1.2.2. Cell ploidy. [Artificially-synthesized and naturally occurring diploids]

Conidia of wild-type isolates of *V dahliae and V.albo-atrum* are usually uninucleate and haploid. This evidence is based on a number of cytological, light and electron microscopy studies (Caroselli, 1957; Heale,1966; Hastie, 1967; Heale *et al.*, 1968; Typas & Heale 1976; Typas & Heale, 1977; Typas & Heale, 1980) and on the high frequency of auxotrophs obtained among conidia irradiated with ultraviolet light (MacGarvie & Isaac, 1966; Puhalla & Mayfield,1974; Clarkson & Heale, 1985a,b,c).

In the Fungi Imperfecti, mutations, heterokaryosis and formation of heterozygous diploids via parasexuality, are responsible for genetic variability and may lead to generations of new pathogenic races (Day, 1960; Tinline & MacNeil, 1969). The term parasexuality was first coined by Pontecorvo (1956) to describe the situation he found in *Aspergillus nidulans*; this involves hyphal fusion and plasmogamy to produce heterokaryons, karyogamy and somatic recombination, which may be followed by non-meiotic reduction of the diploid nucleus to the haploid condition. Variation depends upon the incidence of non-disjunction at mitosis, mitotic crossing over and haploidization. Mitotic crossing over causes recombination of linked genes on the same chromosome, whereas haploidization results in reassortment of genes from different linkage groups (reassortment of whole chromosomes); genes in the same linkage group never show recombination at haploidization (unless mitotic crossing over had previously occurred).

Hastie (1962,1964) studied the parasexual genetics of *V.albo-atrum* by analysis of heterozygous diploids and Tolmsoff (1972) by analysis of homozygous diploids. Hastie (1962,1964) first demonstrated the parasexual cycle as a source of new genetic variability in *Verticillium*. He was able to obtain unstable, heterozygous, prototrophic diploids at a frequency of 10^{-5}-10^{-7} for *V.albo-atrum* from hop (1962) and potato and tomato (1964) from heterokaryons using auxotrophic, complementary mutants, on MM*. He found that these heterozygous nuclei were very **unstable**, yielding two novel genotypes; diploid segregants homozygous for some markers and heterozygous for others, (apparently by **mitotic crossing over**) and a second class of stable haploid, progeny, presumably derived by mitotic nondisjunction (**haploidization**). It is thought that this occurs 'step wise' i.e.: 2n-1, 2n-2, 2n-3.... n. Also haploid segregants were recovered, derived from nuclei that been previously undergone mitotic crossing over. A 3 week-old culture grown from a single diploid conidium yielded 95% haploid conidia, thus haploidization appeared to show a very high frequency in *V. albo-atrum* as compared with *Aspergillus nidulans*. A possible explanation given was the more prolific sporulation of haploid mycelium in segregating cultures of *V.albo-atrum*. Further, Fordyce & Green (1964) demonstrated parasexual recombination obtaining prototrophic diploids through

* minimal medium

interspecific heterokaryons of auxotrophs of *V.dahliae* and *V.albo-atrum*. Hastie (1967) developed a special technique, termed 'phialide analysis', analysing the progeny of each phialide (phialide families); he demonstrated mitotic recombination in phialides of *Verticillium albo-atrum*. Later (1968), the same author analysed the phialide families from heterozygous diploid conidia obtained by crossing two di-auxotrophic haploids of which one parent was also a sooty mutant (so) i.e. carrying a colour marker and demonstrated mitotic recombination in *V. albo-atrum*. Heterozygous diploid conidia yielded a number of phialide families, but families containing only diploid and haploid conidia were absent, showing that haploids were formed not directly from diploids, but rather that diploids formed aneuploids by non-disjunction which finally formed haploids by stepwise chromosome elimination (Hastie, 1968).

Puhalla and Mayfield (1974) first provided evidence of migration and somatic nuclear fusion in an anastomosed cell formed between hyphae of *V.dahliae* strains using phase-contrast microscopy. H-shaped, anastomosed cells were formed between hyphae of *V.dahliae* containing a large nucleus (assumed to be diploid), whereas other anastomosed cells had relatively small nuclei.

A number of studies with UV-produced auxotrophs of *V.dahliae* and *V.albo-atrum* have been performed and interspecific (Fordyce & Green, 1964; Hastie, 1973; Typas & Heale 1976) and intraspecific (Hastie, 1973; Ingle & Hastie 1974; Puhalla, 1973; Puhalla & Mayfield, 1974; Typas & Heale, 1977,1978; MacGeary & Hastie, 1982; Clarkson & Heale, 1985a, 1985b, 1985c) heterozygous diploids have been obtained on minimal media. Hastie (1973, 1978) demonstrated that interspecific diploids, obtained from complementary auxotrophs from *V.dahliae*, *V.albo-atrum* and *V.tricorpus* showed restricted recombination and rare spontaneous hybridization, indicating relative non-homology among these species. Most haploid segregants obtained from these interspecific diploids had parental genotypes, suggesting restricted genetic exchange between populations of these fungi. Recombinant genomes, consisting of part of each original haploid parent genome, are therefore likely to be non-homologous. Glucose/nitrate minimal medium at 26^{0}C favoured the recovery of heterozygous diploids of *V.albo-atrum* (Ingle & Hastie, 1974) while growth at 30^{0}C favoured the recovery of *V.dahliae* heterozygous diploids (Puhalla, 1973). Both of these temperatures represent a limiting condition for normal hyphal growth by haploid strains (except for isolates of the *V.albo-atrum* alfalfa strain which grow well at 26 ^{0}C).

Confirmation of diploidy can collectively be obtained using the following criteria: 1) DNA content, 2) cell volume, 3) nuclear volume, 4) chromosome counts, 5) segregation of parental markers, 6) radiation sensitivity and 7) mutability. There is a close relation between cell size (volume), nuclear volume, DNA content, and ploidy in various organisms in nature. Spore-length among fungi producing mononucleate conidia is a valuable indication of ploidy. Day (1972) found that diploid, triploid and tetraploid sporidia of *Ustilago violacea* were close to 2, 3 and 4 times as long, respectively, as haploid sporidia.

Studies of diploids obtained in the laboratory showed that mature diploid conidia of *Verticillium* were never less than 8.0μm in length (Hastie & Heale, 1984). Hastie (1962) obtained heterozygous diploids from heterokaryons which produced conidia with almost twice the volume of the original, auxotrophic, parental conidia. Further, Puhalla & Mayfield (1974) obtained putative diploids (from heterokaryons between auxotrophs obtained from irradiated conidia of the T9 defoliating isolate of *V.dahliae* from cotton grown at 30^{0}C) which were uninucleate, and with an average conidial size of 11μm

X 4.5μm as compared with 6.4μm X 4.4 μm for the parent isolate T9. Also, the diploid conidia were 1.7X more resistant to killing by ultraviolet light.

Typas & Heale (1976, 1978) obtained heterokaryons between different, but complementary, auxotrophic strains and the conidia from these heterokaryon colonies yielded daughter colonies on minimal media with conidia which were relative longer (6μm-11μm) than those of parental auxotrophs and had a volume 1.8x than those of parental conidia. Feulgen staining showed these conidia to be uninucleate. Conidia produced by heterozygous diploid colonies (from heterozygous diploids synthesized in the laboratory) were **generally unstable** and colonies from these single diploid conidia produce 90%, 95% haploid conidia after three-four weeks of incubation (Typas & Heale ,1977 ; Hastie, 1978, respectively). Typas & Heale (1976a) isolated a strain from tomato that had relatively larger conidia and showed an increased survival on a medium containing acriflavine which was a putative, natural diploid of *V.albo-atrum*. Clarkson and Heale, (1985b) obtained heterozygous diploids from heterokaryons of diauxotrophs of *V.albo-atrum* strains from hop on minimal medium, which, after inoculation in *Antirrhinum majus* yielded four, large-spored isolates; one of these was moderately virulent to hop and remained as a stable diploid up to ten weeks after inoculation in hop. Further, they obtained a recombinant haploid prototroph when the host *Antirrhinum* was inoculated with a pair of complementary diauxotrophic isolates (M18 nic-4, cob-28 and PV3 arg-8, pyr-2) from *V. albo-atrum*, which showed an intermediate pathogenicity between that of the two parental wild types: M18 and PV3, demonstrating genetic recombination with respect to auxotrophic markers and pathogenicity within a non-selective host plant. MacGeary and Hastie (1982) selected haploid (recombinant) prototrophic segregants from heterozygous diploids obtained between complementary auxotrophs, derived from lucerne and tomato strains of *V.albo-atrum*, and showed that 11/21 of them were virulent to both hosts. Further O'Garro and Clarkson (1992) recovered haploid, prototrophic, recombinant isolates from heterozygous diploids obtained from crosses of complementary auxotrophs produced from *V.dahliae* (tomato and eggplant isolates, both race 1 and race 2); and when tested to tomato cultivar Roma (which was susceptible to both races) all were of lower pathogenicity than the wild type parents, except the 'selfed' race 2 X race 2 cross which was as pathogenic as the wild type parent.

Stark (1961) isolated a *V.dahliae* strain from wilted horseradish in Hamburg, Germany, which produced mononucleate conidia which were approximately twice as long as those of *V.dahliae* Kleb., and he named it *Verticillium dahliae var. longisporum*. Along with this isolate, he also isolated, from the same infected root, a typical *V.dahliae* . This strain was investigated extensively by Ingram (1968) who failed to obtain auxotrophs by UV irradiation in a number of attempts (auxotrophic mutations induced in it are concealed by their non-mutant corresponding wild type alleles) and was found to differ from *V.dahliae* (1) in its conidial size, the average spore length being almost twice that of the typical *V.dahliae*, (2) the production of small-spored (haploid) forms by p-fluorophenylalanine (pFa) treatment, (3) induction of auxotrophs only from haploid wild type of *V.dahliae* and not from the large-spored form: *V.d.longisporum* and (4) synthesis of diploid types using complementary haploid auxotrophs which were morphologically indistinguishable from the large-spored, Stark's variety. These latter diploids, as compared with other synthesized diploids, were stable, i.e. they did not produce haploids. Ingram (as stated by Hastie, 1970) synthesised heterozygous diploids from the haploid genomes of '*longisporum*' and from *V.dahliae* or

V.albo-atrum which also showed very infrequent mitotic nondisjunction (haploidization) but mitotic recombination was frequent. Ingram (ibid.), concluded that this (i.e. *V.d.longisporum*) was a true diploid. Later, this was fully confirmed by (a) analysis of conidial size in large population of this isolate using Coulter counter (Typas & Heale, 1977) and (b) by estimation of DNA values by Feulgen DNA microdensitometry by Typas & Heale (1980) who found 25-30 fg DNA / nucleus for haploid conidia and double DNA content for the strain of *V. dahliae* var.*longisporum* as compared with haploid strains of *V.dahliae* and *V.albo-atrum*. Hastie (1970) assumed that Stark's original isolate was an homozygous diploid (arising from the normal haploid form, reported to be parasitizing the same infected horseradish, by either a failure of mitosis or somatic nuclear fusion in a homokaryotic cell). Tolmsoff (1983) extracted DNA from this same isolate and found it to have double the amount (76 fg/conidial cell) of DNA as compared with wild-isolates of *V.dahliae* and *V.albo-atrum*. He also found a range of DNA content (27-45 fg/conidial cell) of mononucleate conidia of 4 wild isolates of *V.dahliae* and 4 wild isolates of *V.albo-atrum* and suggested that wild isolates of *V.dahliae* and *V.albo-atrum* were aneuploids rather than haploids and that instability in culture was associated with heteroploid shifts to new types of aneuploid variants. The same isolate (i.e. *V.d.longisporum*, Stark) was studied by Jackson & Heale (1985), as a reference diploid strain to compare with *V.lecanii* isolates and was found to have almost double the DNA content and conidial volume. Horiuchi *et al.* (1990), in a study based on morphological and enzymatic characters, found the *V.d.longisporum* Stark isolate to have very long conidia (8.9μm), suggesting it was diploid, and later, Baig (1991) haploidized this strain with pFa to produce haploid, small spores. However, the original isolate *V.d.longisporum* Stark proved to be very stable, remaining its diploidy 14 years after its isolation from the diseased horseradish in Germany. This isolate has been used in this study and is referred to as isolate number 195 throughout the thesis.

Similarly, Puhalla & Hummel (1983), in a study of heterokaryon incompatibility in 96 strains of *V.dahliae* from 38 different hosts species, found two isolates from Sweden (one from sugar beet and one from rape) with relatively longer conidia, which did not yield microsclerotia colour mutants after UV treatment and these were assumed to be diploid. Jackson & Heale (1985) confirmed stable diploidy in both these two strains by DNA Feulgen microdensitometry and by conidial sizing. These strains, after treatment with pFa produced segregants with half the conidial volume and relative DNA content of the original parents. Thus, both strains were also assumed to be homozygous diploids and evidence to support this was the observation that all haploid segregants appearing after treatment with pFa were similar in morphology to the parental diploid strain. These two isolates were investigated in this study and are referred to as isolate 161(from sugar beet) and isolate 162 (from rape). Horiuchi *et al.* (1990) also studied these isolates and they were found to have the same morphological and enzymatic characters as isolate 195 (i.e. the original *V.d.longisporum*, Stark). Three other isolates from Brassica crops (two from Chinese cabbage and one from wild radish) in Japan showed the same enzymatic and morphological characters with the three stable diploids above and Horiuchi *et al.* (1990) concluded that these isolates were diploid and they were assigned to *V.d.longisporum*, Stark. In this present study, isolates of *V.d.longisporum* Stark from Chinese cabbage are referred to as 84120, 84013 and the isolate from wild radish as 86207 throughout.

1.2.3. Sub-specific groups in the genus *Verticillium*

Verticillium dahliae Kleb. is an economically important, vascular wilt pathogen of many agronomic, horticultural and landscape plants.

Generally, strains of *V.dahliae* and *V.albo-atrum* are non-host-specific, although some studies of host-specific isolates have been reported e.g. *V.dahliae* attacking Mint/*Mentha* and *V.albo-atrum* attacking lucerne/alfalfa (*Medicago sativa*). The occurrence of distinct strains within a population of isolates attacking a particular host has been reported in Verticillium diseases (Pegg, 1974). Forma/e specialis/es (a sub-specific grouping used for *F.oxysporum* causing vascular wilt, based entirely upon specialized host range) has never been claimed up to the present time although very recently, Griffen, Bainbridge & Heale (in press), suggest the name *V.albo-atrum* var. *medicaginis* for the specialized pathotype attacking alfalfa which is readily distinguished from all other *V.albo-atrum* strains by DNA-fingerprint techniques such as mt-DNA RFLPs & RAPDs as well as by optimum growth at 26^{0}C compared with 21-22^{0}C for all other *V.albo-atrum* strains (Heale, 1985).

Isaac, (1957) described an isolate of *V.dahliae* from wilt diseased Brussels-sprouts from a farm in the Vale of Evesham, UK, which was non-pathogenic to a wide range of plants (hop, tomato, lupine, ester, phlox, broccoli, cauliflower) usually susceptible to attack by *Verticillium* e.g. *Antirrhinum* but pathogenic to Brussels-sprouts; Brussels-sprouts were not susceptible to any other strains or species of Verticillium tested, i.e. *V.dahliae* strains from tomato, sainfoin and phlox; *V.albo-atrum* strains from hop, tomato and potato; a *V.tricorpus* strain from tomato; *V.nigrescens* and *V.nubilum* strains, both from potato. Isaac (ibid.) concluded that this isolate was a distinct **physiological strain** of *V.dahliae*. The same UK isolate assigned as 111 was also used in the present study. Nelson (1950) described a 'microsclerotial form' of *V.albo-atrum* from mint (*Mentha* spp.) which was virulent to mint, but was not-pathogenic to a wide range of other hosts, and *Verticillium* isolates from many other hosts were not pathogenic to peppermint. He considered the mint wilt fungus distinct and misnamed it *V.albo-atrum* (=*V.dahliae*) R. & B. var. *menthae* (microsclerotia form). Later, Horner, (1954) and Fordyce & Green, (1960) also confirmed the host-specialization of these isolates from mint. **Physiological races** based on a single gene for resistance to *Verticillium* in the host have been defined only in isolates of *V.dahliae* from **tomato** (*Lycopersicon esculentum*) which can be distinguished by their ability to cause wilt in cultivars possessing the *Ve* gene as either race 1, or race 2 (Alexander, 1962; Pegg, 1974). Race 2 of *V.dahliae* is pathogenic to *Ve*-containing cultivars and presumably evolved by mutation or by recombination via the parasexual cycle (Heale, 1988). O'Garro & Clarkson (1988) assessed the pathogenicity of race 1 and race 2 isolates of *V. dahliae* in tomato cultivars Roma and Roma VF (*Ve*) and found that race 1 was pathogenic to both cultivars, although Roma was more susceptible.

Pathotypes of *V.dahliae* (sometimes misnamed as microsclerotial forms of *V.albo-atrum*) with distinct levels of pathogenicity have been described in **cotton**. Severe defoliating isolates (T1 or T9) of the pathogen which were highly virulent, causing severe defoliation on several cultivars of cotton, as distinct from non-defoliating pathotypes (SS4), which induce little or no defoliation, have been recognised in N. America since about 1960 (Schnathorst & Mathre, 1966). There are probably two major genes determining

tolerance in the host: *Gossypium barbadense*, and there is also evidence for multigenic 'background' resistance.

Isaac & Keyworth (1947), demonstrated two main types of isolates termed physiological strains of *V.albo-atrum* attacking hop that they differed in their pathogenicity to this host, i.e. fluctuating (mild) isolates were of little economic importance, whereas progressive (severe) isolates frequently killed most of the plants in a hop garden and spread widely.

Sewell & Wilson (1984), found three types of progressive wilt isolates of *V.albo-atrum* from hop (PV1, PV2, PV3), distinguished according to their differential responses shown on a range of different hop cultivars. Clarkson & Heale (1985a) distinguished progressive and fluctuating isolates from hop by the type of foliar symptoms expressed by the susceptible cultivars Fuggle and Wye North-down, and by the rate of re-isolation of the pathogen from basal leaves of inoculated plants.

Isolates of *V.albo-atrum* from lucerne/alfalfa (Isaac & Lloyd, 1959) were reported to be mildly pathogenic to certain hosts such as potato and tomato when experimentally inoculated; conversely, *V.albo-atrum* isolated from hops, potato or tomato were not pathogenic to lucerne, thus alfalfa isolates can be regarded as a distinct physiological strain. This finding has been confirmed in a number of other studies (Heale & Isaac, 1963; Busch & Smith, 1982; Christen & French, 1982) by demonstrating that isolates from different hosts were not pathogenic to lucerne and only isolates from lucerne were pathogenic and economically damaging this host, confirming that isolates of *V.albo-atrum* from this host come from a distinct population. This was also confirmed by vegetative compatibility studies using *nit* (nitrate non-utilizing) mutants by Correll *et al.* (1988); fifteen strains from lucerne/alfalfa from different parts of the world were in the same VCG group viz. VCG01 and were virulent to lucerne, whereas 13 strains of *V.albo-atrum* from several diverse hosts and geographical locations were in a second group viz. VCG02, and were not virulent to lucerne, suggesting that these lucerne isolates were distinct genetically and that they had a common, clonal origin.

1.2.4. Methods for identification and characterization of species and sub-specific groups in the genus *Verticillium*

Prior to the last decade, species of *Verticillium* were characterized primarily on the basis of morphological characters, such as resting structure formation and sub-specific groups were characterized by their virulence to certain hosts. Protein patterns in a disc electrophoretic study and isoenzyme production using isoelectric focusing were examined by Whitney *et al.* (1968), and Carder (1989) respectively distinguishing *V.albo-atrum* from *V.dahliae*. Similarly, a wide range (41) of morphological, physiological and biochemical characters were used to classify 64 isolates of the genus *Verticillium*, especially *V.lecanii* (Jun *et al.*, 1991). Vegetative or heterokaryon incompatibility has been widely used to examine genetic diversity among fungal population, and strains have been subgrouped in vegetative compatibility groups (VCGs). More recent molecular biology techniques, including: Restriction Fragment Length Polymorphisms (RFLPs), and the use of the Polymerase Chain Reaction (PCR) for specific amplification of target DNA using specific primers, or Randomly Amplified Polymorphic DNAs (RAPDs), have been used.

1.2.4.1. Heterokaryosis as a taxonomic tool - vegetative compatibility groups (VCGs)

Heterokaryosis and the parasexual cycle have been used widely as aids to taxonomic classification in the Fungi Imperfecti (Leslie, 1993). The vegetative compatibility system is the mechanism determining the ability of hyphae from two strains of the same species to fuse and form a stable heterokaryon; this has been used to determine the genetic relationship among strains of *V.dahliae* and *V.albo-atrum*. Anastomosis of hyphae and the formation of heterokaryons are prerequisites for genetic recombination; thus, if two isolates cannot form heterokaryons, they cannot exchange genetic information and are in effect genetically isolated. These isolates can be viewed as belonging to distinct populations within a species, belonging to different heterokaryon compatibility groups (Het-c) or vegetative compatibility groups (VCGs). Thus Het-c (Puhalla, 1979) or VCGs (Rowe, 1994) are defined as sub-specific groupings within the same fungal species, in which hyphae of isolates can fuse to form heterokaryons and exchange genetic information. Conversely, isolates that are incapable of anastomosing and fail to form heterokaryons, are referred to as being vegetatively incompatible with one another and therefore belong to different VCG groups.

The first work in identifying heterokaryon compatibility groups in *Verticillium dahliae* was performed by Puhalla (1979) who used morphological mutants, microsclerotia-pigment colour mutants (alm: hyaline microsclerotia and brm: brown microsclerotia) induced by exposure to UV irradiation. He classified 19 isolates into 4 subgroups (P1, P2, P3 and P4) according to their ability to form a line of black microsclerotia between paired brm (red/brown mutant) and albino/hyaline (lacking pigmented microsclerotia) mutants. All defoliating isolates of *V. dahliae* to cotton were assigned to Group P1. Later, Puhalla & Hummel (1983) used the same technique to classify 96 isolates of *V.dahliae* from 38 different host-plants and from 15 countries identifying 16 heterokaryon compatibility groups (86 isolates classified).

Later, Joaquim & Rowe, (1990) using, nitrate-nonutilizing (*nit*) mutants, selected from wild-type strains on a medium containing potassium chlorate, classified 22 strains that had been previously assigned to 15 heterokaryon groups using microsclerotia colour mutants (Puhalla and Hummel, 1983) in only 4 VCGs. They also did complementation tests between *nit* mutants derived from 21 additional strains of *V.dahliae* and confirmed the existence of these 4 VCGs. Pairing of nit mutants in Petri dish assays were conducted by placing a *nit* 1 (incapable of utilising nitrate, but able to use nitrite, ammonium, hypoxanthine and uric acid) or *nit* M (incapable of utilizing nitrate and hypoxanthine but capable of utilizing the remaining three other nitrogen sources mentioned above) derived from one strain at the centre of a plate with MM, and the two nit mutants (*nit* 1, *nit* M) of the tester strain on either side. Heterokaryon formation was detected when prototrophic growth developed at the hyphal interface of the nit mutant of the centre and/or both nit mutants of the tester strain. Isolates considered to be incompatible when microsclerotia colour mutants were employed were nevertheless compatible when *nit* mutants were used. The same conclusion was reached Strausbaugh *et al.* (1992) who tested 26 strains of *V.dahliae* originally assigned to 16 VCGs by Puhalla. They assigned 3 to VCG 1, 13 to VCG 2, 7 to VCG 4 and 1 to a newly defined VCG 5. California isolates from potato from one location in California were assigned to VCG1 while others collected from another location in California were assigned in VCG 4. Earlier, Heale (1988) had given a possible explanation of this phenomenon when trying to explain the conflicting results of heterokaryon incompatibility data of Puhalla (1979), Puhalla & Hummel (1983), and the lack of any

evidence of clear-cut incompatibility as observed by Typas & Heale (1976) and Clarkson & Heale (1985b) using complementary auxotrophs. The use of paired brown and albino microsclerotia colour mutants to assess compatibility, probably requires many hyphal fusions to produce a visible line of black microsclerotia (indicating the heterokaryon reaction); thus, very rare hyphal fusions between moderately incompatible isolates could be overlooked by the microsclerotia test of Puhalla & Hummel (1983), thus overcoming incompatibility, and therefore to an apparently, much larger number of compatibility groups by Puhalla test.

Further, in a second paper, Joaquim & Rowe (1991) used the same VCG system with nit mutants to classify 189 strains of *V. dahliae* isolated from potato plants and soil from 22 Ohio potato fields. Two of these strains were assigned to VCG 1, 53 to VCG 2 and 128 to VCG4. They subdivided the VCG 4 group to VCG 4A and 4B, according to their different reactions when paired against both VCG 4 tester strains. These strains were more virulent to potato and interacted synergistically with the root lesion nematode *Pratylenchus penetrans* (Botseas & Rowe, 1994).

Strausbaugh (1993) also used the *nit*-mutant system to characterize isolates of *V.dahliae* obtained from infected potato plants in Idaho, which were all assigned to VCG 4. Chen (1994), in a study of 42 different strains of *V.dahliae* from woody ornamental plants using the *nit*- mutant system, assigned 30 of them to VCG 1, 2 to VCG 2, and 4 to VCG 4. Thus, a very narrow genetic diversity was found among these strains, even though they were from a broad plant species-host range. Nagao *et al.* (1994a), using the same *nit*-mutant system, examined 70 isolates of *V.dahliae* collected from Japan. They recognised three main groups: VCG JI, VCG J2 and VCG J3, which corresponded to the three pathotypes previously reported according to 5 differential hosts viz. eggplant (except one isolate), tomato and sweet pepper (except one isolate) respectively. They also tried to obtain *nit* mutants from diploid isolates i.e.. *V.dahliae* var. *longisporum*, Crucifer Group D (Horiuchi *et al.*, 1990), but this was not possible; these latter isolates did not sector on the medium contained chlorate. Dr. Portenko, Rape Institute, Lipetsk, Russia (personal communication) was also unable to obtain *nit* mutants from diploid strains i.e. *V.d.longisporum* isolated from oilseed rape plants. Portenko *et al.* (1994) had earlier studied vegetative compatibility in 24 strains of *V.dahliae* from cotton which were differentiated according to their virulence in cotton cultivars, and 33 strains of *V.dahliae* from seven different host species using *nit* mutants. All isolates from cotton were in the same VCG group (VCG1, described in Joaquim & Rowe, 1990), showing diversity in virulence within a single VCG; the other 33 strains from different hosts were assigned: 31 in VCG1, one from cotton in VCG2 and one from strawberry in VCG3.

1.2.4.2. Identification of *Verticillium* species and sub-specific groups using Restriction Fragment Length Polymorphisms (RFLPs)

Total genomic DNA, or specific DNA fractions, can be extracted from fungal material and digested with restriction endonucleases that cut DNA strands at certain nucleotide sequences; thus, differences in the original sequence of DNA are reflected in restriction fragment length polymorphisms [RFLPs] (Michelmore & Hulbert, 1987) that are demonstrated according to band sizes after separation in agarose gels. Usually, for most organisms the RFLP patterns are too complex to allow comparative studies of different isolates,

and for this reason, DNA bands are transblotted onto a nitro-cellulose membrane and labelled with probes (Southern blots). Probes for detection of RFLPs can be randomly generated genomic probes (from the total genomic DNA or excluding mitochondrial DNA), ribosomal DNA (rDNA) or mitochondrial DNA (mt DNA).

Typas *et al.* (1992) hybridized digested genomic or mitochondrial DNA (with restriction endonucleases *Eco*RI or *Hae*III) from 7 different species of *Verticillium* (*V.dahliae, V.albo-atrum, V.tricorpus, V.lateritium, V.lecanii, V.nigrescens* and *V.nubilum*) using probes based on the rRNA gene complex of *V.albo-atrum*, or a clone containing the large rRNA sub-unit from mt-DNA of *Podospora anserina.* DNA digested with *Hae*III gave distinct mt-DNA patterns with all seven *Verticillium* species and the mt-DNA probe hybridized to a distinct band for each species. This analysis of mt-DNA also resulted in lucerne/alfalfa isolates of *V.albo-atrum* being distinguished from non-lucerne isolates of the pathogen.

Carder & Barbara (1991) used 28 random genomic clones from a partial library of a highly virulent isolate of *V.albo-atrum* from hop, rDNA from flax and mt DNA from *V.albo-atrum* as probes in southern blots of genomic DNA digested with *Eco*R1 to differentiate 6 different species of *Verticillium*: *V.dahliae, V.albo-atrum, V.lecanii, V.tricorpus, V.nubilum* and *V.nigrescens. Eco*R1 digested DNA separated on agarose gel gave species-specific banding patterns, allowing the differentiation of *V.dahliae* from *V.albo-atrum* (a 2Kb band was present in *V.dahliae*, but was absent in *V.albo-atrum*). Further, one random genomic probe successfully differentiated *V.dahliae* from *V.albo-atrum* and also isolates from *V.albo-atrum* from lucerne/alfalfa were clearly differentiated from all other *V.albo-atrum* isolates. This probe did not hybridize with the other *Verticillium* species. No molecular differences and limited variation was found within the three lucerne/alfalfa isolates and the 'non-lucerne' isolates tested respectively. Within isolates of *V.dahliae*, there was greater variation but only limited indication of sub-specific groups. The use of probes for either rRNA gene coding regions or the mitochondrial genome produced patterns that were of little used in discriminating *Verticillium* species.

The same co-workers (Okoli *et al.*, 1993) used 71 random genomic clones from a partial genomic library from *V.dahliae* from strawberry to hybridize DNA from 17 isolates of *V.dahliae* digested with *Eco*R1 and *Hind*III; 15 isolates fell clearly into either of two groups: Group A and Group B, and the other two isolates (I-isolates, intermediate) showed polymorphisms not associated with the major A/B differentiation. They also tested 13 more additional isolates from soil and nine fitted into Group A and four into Group B. There was no apparent correlation between the major RFLP groups and the original host of the isolate or geographical association, but RFLP groups were correlated with cellulase isoenzyme pattern. In another paper, the above authors (Okoli *et al.*, 1994) used three recombinant clones as probes; one from a partial genomic library of DNA from *V.albo-atrum* (pVA), one from a partial genomic library of DNA of *V.dahliae* (pVD), and one which contained the flax ribosomal (rRNA) gene repeat (pRE1), to hybridize genomic DNA digested with *Eco*R1 and *Hind*III from host-adapted, haploid *V.dahliae* isolates from peppermint and host-adapted diploid isolates (i.e. *V.dahliae* var. *longisporum* Stark) from Brassicas. The isolates from peppermint formed a host-specific group (Group M), equivalent in status to the sub-specific groups A and B, while diploid isolates from *Brassica* species formed another markedly distinct group (Group D) from all the other haploid isolates in the other three *V.dahliae* sub-specific groups (A, B, M) and from *V.albo-atrum.* Using the same technique, and probes, Carder & Barbara (1994) found that

only two isolates from one group, Group JD, diploids, which were only pathogenic to turnip [one of the four pathogenicity groups defined by Horiuchi *et al.* (1990)], corresponded directly with the existing RFLP Group D, mentioned above. The other Japanese isolates could not be placed in any of the previous defined RFLP groups. The RFLP Group D of the diploids was only distantly related to the six haploid isolates tested.

1.2.4.3. Use of PCR to amplify specific or random DNA sequences to identify species and sub-specific groups of *Verticillium*

The polymerase chain reaction provides a simple method to exponentially amplify DNA sequences by *in vitro* DNA synthesis. This includes three essential steps: Melting (denaturing) of the target DNA into two strands, annealing of two oligonucleotide primers (specific amplification) or one oligonucleotide primer to the denatured DNA strands, extension and synthesis of new double strands using a thermostable DNA polymerase, *Taq* DNA polymerase from *Thermus aquaticus*. These newly-synthesized DNA strands serve as templates for subsequent DNA synthesis and the three steps are repeated automatically in a thermocycler up to 50 times.

Genes encoding cytoplasmic ribosomal RNA (rDNA) have been widely used as probes in taxonomic analysis of many organisms and microorganisms. The number of genes encoding rRNA range from 60 to 220 copies per haploid genome (Cassidy *et al.*, 1984; Russell *et al.*, 1984). The large, multiple, copies of the rDNA transcription units are clustered in long, direct, tandem arrays. Coding regions 18S, 5.8S and 28S are grouped in that order in a single RNA polymerase I transcription unit and separated from another by an intergenic spacer (IGS) consisting of a non-transcribed spacer region (NTS) and external transcribed spacer region (ETS). The entire transcribed unit includes two, non-coding, spacer DNA segments, internal transcribed spacers (ITS) that separate the three coding regions from one another (ITS1 and ITS2), see Figure -1.4-.

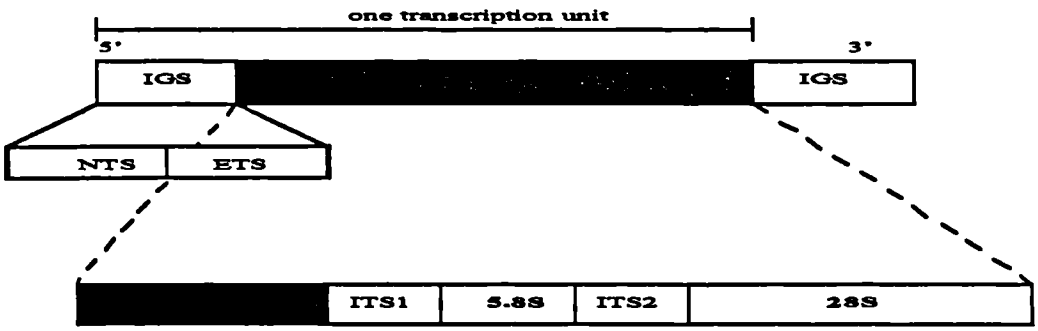


Figure-1.4- Diagram (after Nazar *et al.*, 1991) of a single transcription unit of rRNA consisting of the 18S, 5.8S and 28S transcribed regions which are connected via spacer sequences. The intergenic spacer region (IGS) is found between each repeat, which consists of a non-transcribed spacer (NTS) and an external transcribed spacer (ETS). There are also two internal transcribed spacers ITS1 and ITS2. (not to scale).

The coding regions have been found to be highly conserved which is in marked contrast with the non-coding spacer regions showing a considerable sequence variation, (Verma & Dutta, 1987). The internal transcribed spacer (ITS) regions show a considerable sequence variation in a number of fungi (Lee & Taylor, 1992; Kim *et al.*, 1992; Xue *et al.*, 1992; Yao *et al.*, 1992) including *Verticillium* (Nazar *et al.*, 1991) and even more sequence differences are found in the IGS regions between the rRNA gene repeat units (Henson & French, 1993).

Nazar *et al.* (1991) isolated and sequenced the transcribed spacer region between the mature 18S and 28S units of rRNA from *V.dahliae* and *V.albo-atrum* from lucerne/alfalfa, and found a cluster of 3 non-homologous nucleotides in the ITS1 and a cluster of 2 non-homologous nucleotides in the ITS2 regions. From these sequenced regions they synthesized complementary oligonucleotide primers for PCR amplification of fungal DNA from infected host plants. The corresponding primers were used successfully to differentiate the two species of *Verticillium*. Using the same specific primers for *V.dahliae* and *V.albo-atrum*, Xu *et al.* (1993) prepared homologous (with partial deletion of the primer template) and heterologous (unrelated sequences from *Fusarium oxysporum*) internal control templates which were amplified in the same reaction with the experimental samples from infected material to allow accurate quantification of the living fungus in the infected tissue. The same group (Moukhamedov *et al.*, 1994) sequenced the ITS1 and ITS2 regions of the ribosomal genes of *V.tricorpus* from cotton, and designed a primer set that specifically identified this species of *Verticillium*. *V.tricorpus* was found to be more divergent. Seventeen and twelve base differences were identified in these regions collectively when *V.tricorpus* was compared with *V.dahliae* and *V.albo-atrum* respectively. *V.albo-atrum* and *V.tricorpus* shared the same 5 nucleotides that *V.dahliae* lacked, thus indicating *V.tricorpus* is more closely related to *V.albo-atrum* than it is to *V.dahliae*. Using *V.albo-atrum*, *V.dahliae* or *V.tricorpus*-specific oligonucleotide primers, Robb *et al.* (1993), found an isolate (110) of *V.albo-atrum* from potato which responded negatively with all the three primers above. Sequencing of the ITS regions of this unusual isolate (110) revealed that it was more similar to *V.tricorpus* than to all other isolates of *V.albo-atrum*. In the same study, using a primer set designed for this unusual isolate, they found other 12 isolates from potato from Prince Edward Island (Canada) to react positively with these primers. They found two sub-specific groups, one *V.albo-atrum* 1 group which reacted positively with the original set of primers and a *V.albo-atrum* 2 group which reacted positively with primers designed from the above unusual isolate (110) from potato. They also found that the unusual isolate Luc2 from alfalfa UK (but avirulent to alfalfa) was also in the group *V.albo-atrum* 2. Morton *et al.* (1995) determined the sequences of the ITS1 and ITS2 of the rRNA genes of 38 *V.albo-atrum* and *V.dahliae* isolates and one isolate of *V.tricorpus* from PCR products using consensus rRNA gene primers for these regions. The majority of the haploid *V.dahliae* isolates were identical and were separated from the lucerne isolates (L) of *V.albo-atrum* only by 5 base pair changes. The 'non-lucerne' isolates (NL) of *V.albo-atrum* were also identical and different only in one base pair from the L group of *V.albo-atrum*. The four diploid isolates (i.e. *V.d.longisporum*) tested (two from Sweden: 161, 162 and two from Japan: 84120, 84013) [all included in the present study] were identical and separated from the two identical L *V.albo-atrum* isolates by only one base pair in these ITS regions.

Random amplification of polymorphic DNAs (RAPDs, pronounced 'rapid') is an *in vitro* amplification of genomic DNA [polymerase chain reaction (PCR)] by single short oligonucleotide primers

(10-20 bases) of arbitrary nucleotide sequence and under non-specific conditions (annealing at 30-40^{0}C), which generates a number of patterns of anonymous polymorphic DNA fragments or fingerprints, (Welsh &MacClelland,1990; Williams *et al.*, 1990) which can be analysed directly on agarose gels stained with ethidium bromide and can be used for genetic mapping and strain identification. This is a very powerful technique for identifying closely related strains with similar phenotypes, but with different other properties such as pathogenicity (Bainbridge, 1994). The main advantages of this technique are: 1) It does not require prior knowledge of the DNA sequences to be targeted. 2) Small quantities of the target DNA are required. 3) The target DNA does not need to be pure. 4) It is very rapid and not very expensive. 5) There is no need for radioactive probes and is therefore safer to handle.

RAPDs were used in this investigation for strain characterization and to assess genetic diversity among and between haploid isolates of *V.dahliae* of world-wide origin and different hosts and isolates of *V.dahliae* (either known to be *V.dahliae longisporum* or putative isolates of *V.dahliae longisporum*) of world wide origin from Brassica crops. The original diploid isolate 195 of *V.d.longisporum* described by Stark was also used in this investigation along with the two verified diploid isolates 161, 162 (i.e. known to be *V.dahliae longisporum*) from Sweden (Jackson & Heale, 1985). Two isolates of *V.albo-atrum*, one from chrysanthemum (230) and one from lucerne/alfalfa (234) were also used as representative isolates of this species.

1.3. Verticillium wilt of Brassica- and related species

Verticillium wilt of winter oilseed rape (*Brassica napus* subsp. *oleifera*) had been reported to be caused by a pathogen previously identified as *Verticillium dahliae* Kleb. (Krikun & Bernier, 1987; Seidel & Zeise 1990). It is a serious problem involving severe economic losses in Sweden since 1960 (Svenson & Lerenius, 1987). In Germany, Verticillium wilt of oilseed rape occurs in the whole country but mainly in the North (Krüger, 1989). There are also indications of isolated outbreaks in France the last ten years (Dr. Hortense Brun, INRA, Le Rheu, personal communication), Poland (Sadowski *et al.*, 1995; Zielinski & Sadowski, 1995), southern Russia and The Ukraine (Dr. L. Portenko, personal communication). Krikun and Bernier (1987) reported symptoms caused by *V.dahliae* in Canadian oilseed rape and mustard after experimental inoculation with two isolates of the fungus from potato and pea respectively, but there have been no reports of any disease outbreaks in rape in Canada at that time or since. The disease has not yet been reported from the UK, even though oilseed rape is widely grown here. Previous attempts by the present author to re-isolate *V.dahliae* from debris that had been kept overwinter from field pots and oilseed rape farm fields where the crop was known to be infected with *Leptosphaeria maculans* in NIAB, Cambridge, failed. It seems therefore that the pathogen has not yet spread to Britain, but a potential threat exists in view of the very large area of the crop now cultivated here, and the susceptibility of oilseed rape and Brassica related species that has been demonstrated under experimental conditions in this laboratory (Baig, 1991). Apparently, the only known report of an outbreak of Verticillium wilt in any Brassica crop caused by *V.dahliae* in the UK, was an isolated event reported for Brussels sprouts in the Evesham area during 1957 (Isaac 1957).

Baig (1991) tested 11 isolates of *V.dahliae*, *V.albo-atrum* and *V.tricorpus* (6 from Cruciferous-hosts and 5 from non-Cruciferous-hosts) against 8 cultivars of oilseed rape; he found only 4 isolates of *V.dahliae* (=*V.dahliae longisporum* Stark) to be pathogenic to rape. The same isolates were also pathogenic to other Cruciferous-hosts i.e. cabbage, Brussels sprouts, Chinese cabbage, Swede, Japanese wild radish; *Arabidopsis thaliana* (ecotype: Landsberg) also exhibited external symptoms when inoculated with these 4 isolates. Kroeker (1976), performed inoculation tests with oilseed rape isolates of *V. dahliae* on broad bean, garden peas, potatoes, soybean, sunflower and lucerne and found all them (under such experimental conditions) to be susceptible to this pathogen, except lucerne (which is only attacked by a specialized host-pathotype of *V.albo-atrum*) (Heale & Isaac, 1963; Bush & Smith, 1982; Christen & French, 1982; Correll *et al.*, 1988; Typas *et al.*, 1992; Griffen, Bainbridge & Heale , in press).

The fungus infects the plant through the roots (via direct penetration of the epidermal cells or via open wounds) and transverses the cortex and endodermis to enter the vascular system; it then moves upwards in the vessels largely by production of 'bud spores'. The pathogen grows within the plant by the extension of mycelium and the flow of spores in the xylem of vessels. Niederleitner *et al.* (1991) studied the ultrastructural responses of oilseed rape and *Impatiens balsamina* (Balsaminaceaea, chosen as a non-Cruciferous universal host) plants artificially infected with an isolate of *V.dahliae* (=*V.d.longisporum*) from oilseed rape resulting in a different development of symptoms; in oilseed rape only yellowing of the leaves occurs whereas in *I.balsamina* a true wilt occurs. They described occlusion of xylem vessels by

tyloses and fibrillar material in both species although apposition layers on oilseed rape were thinner than those observed in *I.balsamina*. They observed coating material on xylem vessels and hyphae and also degenerated membranes, cell walls and organelles indicative of toxic and enzymatic activities by the fungus.

A marked reduction of yield of oilseed rape in the field due to the disease maybe due to a number of causes; thus it can involve a complex of several different fungi: *V.dahliae* (=*V.d.longisporum*), *Phoma lingam*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* according to Kroeker (1976). Verticillium infections of winter-sown oilseed rape seedlings begin immediately after germination in the Autumn, although the symptoms appear much later in June & July (Seidel & Zeise, 1990), but are frequently difficult to distinguish from senescence. Premature ripening of oilseed rape is associated with *V.dahliae* (Dr. Zeise, personal communication, Brun & Jacques, 1991) and symptom development and expression is related to the later maturity of the plant, when there is a major competition for nutrients and not earlier, as demonstrated by Seidel & Zeise (1990) who used rapid cycle Brassicas and demonstrated symptoms during the 40 days life cycle of these plants. The best criterion of infection and scoring of the disease is the black discolouration on the stem and the formation of black microsclerotia by *V.dahliae longisporum* within the subepidermal/outercortical layers of the dying tissues of the plant (see Results section). Verticillium wilt causes yield reduction by the premature ripening that causes seed scattering and loss (during harvesting particularly), and by reducing the single plant yield. Field experiments showed that Verticillium disease also reduced the size of the individual seeds (Zeise, 1992). The most important factor determining the extent of the attack appears to be the amount of inoculum (microsclerotia) in the soil and the control of the disease demands many years without susceptible crops and weeds (Berg, 1984). According to an investigation in Rostock, Germany, (Zeise & Seidel, 1990), by increasing oilseed rape in the rotation to 33%, the number of plants killed by *V.dahliae* increased from 2% to 56% during 5 years. In Scotland, where the disease is absent, growers sow oilseed rape in 3 consecutive years, followed by 2 years of cereals and then again 3 years of oilseed rape. Crop rotation in disease areas ought to allow at least 5-6 years between rape crops to minimize the losses and prevent build-up of the inoculum. Benomyl has increased the yield of oilseed rape in wilt-infested soils by 10% (Kroeker, 1976). Up to now, the oilseed rape cvs which are mostly used in Germany show very little difference in resistance to *V.dahliae* (Krüger, 1989). Plant breeders are interested in selecting oilseed rape varieties resistant to Verticillium; in Göttingen (Germany), they have attempted to use 'early selection methods' by quantifying the fungal biomass (determination of fungal ergosterol, determination of different enzyme activities, ELISA) in plants a few weeks after artificial inoculation (Dr. Holtshulte, personal communication). Additionally, in Germany, Zeise (1992) has used a screening test that favours the development of *V.dahliae* in oilseed rape plants and rapid expression of symptoms (the plants are grown under stressed conditions). This test has revealed a number of cultivars that showed some differences in susceptibility/resistance, and now it is in use in a number of oilseed rape breeding stations in Germany for early selection of genotypes with reduced susceptibility to *V.dahliae* (=*V.d.longisporum*). Zeise (1995) tested the virulence of 30 *V.dahliae* isolates from 11 different hosts against 3 important crops: oilseed rape, tomato (*Lycopersicon esculentum*) and linseed (*Linum usitatissimum*) and found that these plants were differential hosts for the above isolates.

A similar Verticillium disease problem occurs in other Brassica crops such as Chinese cabbage

(*Brassica campestris* var. *pekinensis*), Japanese radish (*Raphanus sativus*) in Japan (Yui *et al.*, 1985; Horiouchi *et al.*, 1990) and in various Brassica crops in China (Yen-Yun, personal communication). Horiuchi *et al.* (1990) classified Japanese isolates of *V.dahliae* in 4 different groups i.e. Group A: isolates pathogenic to eggplant and turnip; group B: isolates pathogenic to eggplant, tomato and turnip; group C: isolates pathogenic to eggplant, sweetpepper and turnip and Group D, isolates only pathogenic to turnip. Isolates that were only pathogenic to turnip had been isolated only from Cruciferous hosts and showed morphological similarities with the diploid isolate *V.d.longisporum* Stark (1961) and the two diploid (Jackson & Heale, 1985) isolates, one from rape and one from sugarbeet, from Sweden, and for this reason they were considered to be *V.d.longisporum* Stark. Ciccarese *et al.* (1987) reported severe outbreaks of Verticillium wilt on *Cichorium intybus* (chicory) and *Brassica rapa* (broccoli raab) in Southern Italy. Pathogenicity tests performed with 4 isolates named as *V.dahliae*, 2 from infected chicory plants, one from infected broccoli and one from diseased tomato, showed that chicory was only susceptible to chicory isolates. In a study of recovery of *Verticillium dahliae* from weeds in Farmers' fields in Peru, 3 species representing 3 Cruciferae genera (i.e. *Brassica campestris*, *Capsella bursa-pastoris* and *Diplotaxis muralis*) were hosts to *V.dahliae*, and when experimental tests were conducted with one isolate from an "unknown" weed from the same field that was found to be most virulent to potato, the above first two host species were susceptible and showed external symptoms (Vargas-Machuca *et al.*, 1987). Blush *et al.*, (1978) also previously reported *Capsella bursa-pastoris* to be infected by *V.dahliae* after artificial inoculation. *V. dahliae* is also the primary causal agent (*Fusarium roseum* and *Pseudomonas fluorescens* are the others) of the root rot complex disease of horse radish (Muller *et al.*,1982; Percich &Johnson 1990, Eastburn & Chang, 1994) that causes black vascular discoloration in naturally and artificially infected plants. This disease was first described in Germany as early as 1895 and the causal agent was first identified as *V.dahliae* Kleb. later in 1924, (Blattny, 1928; Gram & Rostrup, 1924). Chang & Eastburn (1994) tested the susceptibility of seven species of plants previously reported to be hosts to *V.dahliae* i.e. Brussels sprouts, eggplant, horseradish, pepper, sunflower, tomato and watermelon to a strain of *V.dahliae* from horseradish, and they also tested the virulence of 11 strains of *V.dahliae* on horseradish; they found that only the horseradish, eggplant, potato and sunflower plants showed external symptoms typical of Verticillium, and horseradish was most susceptible to all strains tested, although the horseradish strain of the pathogen was proved to be the most virulent isolate to this host. In 1990, five commercial cauliflower (*Brassica oleraceae* L. var. *botrytis* L.) fields in coastal California in the United States were reported to be affected by *V.dahliae;* by 1992, twelve more fields were also reported to be affected and the disease had become a significant threat to cauliflower production (Koike S. *et al.*, 1994). Experimental pathogenicity tests by the same author, showed that all commercially available cauliflower cultivars, and also other Brassicas: Chinese cabbage (*B. pekinensis*, Lour.), cabbage (*B. o. capitata* L.) and bok choy (*B. chinensis* L.) were susceptible to isolates isolated from infected cauliflower plants. Broccoli (*B. o. botrytis*), a close relative of cauliflower, although exhibiting occasional blackening of the vascular system, did not show any leaf chlorosis, defoliation, wilting or stunting, as observed with the other Brassica hosts tested and the fungus was never isolated from this host. Verticillium wilt of cauliflower has also occurred recently in the Netherlands, Germany and Japan (Koike S. *et al.*, 1994); in France, *V.dahliae* has been re-isolated from

cauliflowers grown intensively in plastic tunnels for seed production in Finnistaire, France during 1990 (Dr. Jeremy Sweet, personal communication).

1.4. Non-host resistance, species-specific resistance, and basic compatibility

Non-host resistance (basic resistance, general resistance, broad resistance) is a defence reaction of a plant species towards a pathogen that normally is not considered to be pathogenic for this host. This type of resistance is parasite non-specific as seems very unlikely that every plant has parasite-specific resistance genes for all potential fungal or other pathogens (Heath, 1981). It is expressed by all members of the plant species to unadapted inducers involving constitutive (non-active) defences [preformed physical barriers or chemical deterrents in the plant tissue], or induced (active) plant defences triggered by non-specific fungal factors, known as **elicitors** (e.g. fungal cell wall components). An example of elicitation has been reported by Davis *et al.* (1993) who isolated a glycoprotein from *V.dahliae* and showed that its protein portion induced phytoalexin synthesis in cotton and soybean cell suspension cultures while the polygalacturonic acid fraction induced an oxidative burst.

It is believed that this system is complex physiologically and genetically, involving many genes in the plant (Heath, 1991). The ability of a fungus to successfully parasitize a host plant will depend upon it avoiding (non-recognition), actively suppressing, or overcoming, these preformed or/and induced non-specific defence mechanisms such as detoxification, tolerance, toxin production, suppressors (Heath, 1981). Further selection pressure on the plant by the compatible pathogen leads to **cultivar resistance** (one step, one gene) where defence mechanisms are switched on when the plant recognizes a specific feature of the pathogen. Thus the resistance (R) genes in the plant host recognise specific pathogen features determined by *avr* genes of the pathogen [gene-for gene concept, (Flor, 1955; 1971)]. Further selection pressure on the pathogen by the plant due to their resistance leads to new pathotypes that overcome cultivar resistance and lead to **cultivar susceptibility**. The model of basic compatibility suggests that the cultivar resistance is superimposed on basic compatibility and is due to selection pressure by the pathogen. All the above steps are a consequence of the prolonged coevolution of biotrophic pathogens and their hosts; pathogens causing vascular wilts (*Fusarium* and *Verticillium* species) are semi-biotrophs (or hemi-biotrophs) and appear far less evolved than the true biotrophs. The only two well documented examples of single dominant genes in host plants controlling recognition events in vascular wilts are: 1) the *Ve* resistant gene in tomato that confers resistance to Race 1 of *V.dahliae* (Alexander,1962) and *V.albo-atrum* (Schaible *et al.*, 1951) and 2) the *I* gene in the same host (Bohn & Tucker, 1939) that confers resistance to *Fusarium oxysporum fsp. lycopersici*, both of which apparently control the rate and the extent of occurrence of a number of defence responses (wall appositions, coatings, gels, tyloses, lignification, suberinization, phenolics, phytoalexins, etc.) that are multigenically-determined (Beckman, 1987). Selection pressure on Race 1 by tomato cultivars carrying the resistant gene *Ve* led to the comparatively late appearance of virulent isolates of Race 2, indicating that this is probably a multiple step process Heale (1989).

Several models have been proposed to explain the mechanisms regulating specificity in plant/pathogen interactions. Non-specific elicitors released by potential parasites may be recognized by the host and trigger defence responses. Compatible pathogens become successful pathogens by the production

of specific suppressors that negate the action of the non-specific elicitors, thus inhibiting triggering of resistance.

Robb *et al.* (1987) provided evidence to support the view that the *Ve* gene in tomato that confers resistance to *V.albo-atrum*, confers resistance to the suppressive effect of a suppressor, that suppresses at least the coating material production in the colonized vessels. Evidence for that was that in susceptible plants (lacking the *Ve* gene), the secretion of coating material was delayed in colonized vessels containing original trapping sites, but was initiated in surrounding, uncolonized vessels; furthermore, the coating response was also initiated by non-pathogenic root microflora in susceptible plants (Robb *et al.*, 1987). Thus, susceptible plants are vulnerable to a pathogen specific factor (suppressor) that suppressed coating responses which otherwise would confer resistance. In susceptible tomato plants, there was a much lower elevation of PAL (phenylalanine ammonia lyase) activity as compared with resistant plants, as well as suppression of mRNA levels, suggesting that the pathogen suppresses defence genes in susceptible plants (Lee *et al.*, 1992a). Furthermore, race 2 of *V.dahliae* was found to suppress one of the defence responses (coating) in resistant tomato plants carrying the *Ve* gene for resistance to race 1, thus becoming a successful colonizer (Gold & Robb, 1994).

1.5. Host-parasite responses to vascular wilts. Compatible and incompatible interactions

Root exudates stimulate the germination of the resting microsclerotia of *V.dahliae* and the resting mycelium of *V.albo-atrum* producing hyphae and conidiophores which produce abundant conidia, either of which can initiate the infection (Schreiber, 1963; Schreiber & Green 1962). The pathogen enters the plant through natural or artificial wounds or intact epidermis/root hairs of young roots, transverses the root tissues (intercellularly and intracellularly) and is oriented towards the xylem (Bishop & Cooper, 1983a, 1983b, 1984; Elango *et al.*, 1986). After penetrating the endodermis and entering the xylem vessels it starts to sporulate (production of blastospores/budspores). After successful colonization of the root and shoot xylem, the expression of wilt symptoms occurs in the host plant. Thus, two sets of event have to take place before the development of Verticillium wilt. The pathogen must 1) gain entry into the vascular system of the host, so it has to transverse the extravascular elements (epidermis, cortex and endodermis) and 2) continue to colonise the xylem tissue extensively and intensively, so it has to overcome constitutive and induced barriers in the xylem.

Mechanisms of wilt resistance can be divided into :

1. Mechanisms that deter or prevent the invasion into the vascular cylinder, primary determinative phase.(Beckman, 1989). All extravascular tissues (epidermis , cortex, endodermis) have a high level of resistance to wilt pathogens and this is maintained by the general defence strategy of constitutive and induced lignification of epidermis and cortex (which is less effective as the plant matures) as well as suberinization of the endodermis (which increases with age) (Talboys, 1958). Lignitubers, peg-like structures develop actively due to the thickening of the primary cell wall by addition of structural material on the inner surface and around the penetration hyphae (Griffiths, 1971). They are infused with lignin, thus becoming very resistant to penetration. Bishop & Cooper (1983a, 1983b, 1984) studied ultrastructurally

the root invasion of resistant and susceptible tomato and pea cultivars by three vascular pathogens (*Fusarium oxysporum* f.sp. *lycopersici*, *F.ox. f.sp. pisi* and *V.albo-atrum*) and found that epidermal and cortical cells of cvs in compatible and incompatible interactions responded similarly, by the formation of **wall appositions** adjacent to hyphae and formation of lignitubers termed **papillae** (suggested to result from enlargement of primary appositions) that **lignified** and thus restricted hyphal growth (complete penetration of papillae by hyphae was not observed). The endodermis was the only layer that showed a differential reaction between compatible and incompatible interactions by accumulation of e-opaque and autofluorescent material within the colonized resistant endodermal cells, and adjacent intercellular spaces, as well as by wall apposition on adjacent stellar cells, all leading to a reduction of the number of hyphae that entered the stele.

Hypersensitivity responses (**HR**) (Tjamos & Smith , 1974) and accumulation of host secondary metabolites (phenolics, terpenoids, flavanols, acetylenes), or **phytoalexins** , are also associated with resistance to root invasion of epidermal and cortical cells of tomato inoculated with *V.albo-atrum*. Hydrolytic enzymes, **ß-1,3-glucanases**, were found to accumulate earlier in root cells in incompatible interactions involving *Fusarium oxysporum* f.sp. *radicis-lycopersici* and a resistant tomato cv. as compared with compatible interactions involving *F. ox. f.sp. radicis-lycopersici* and a susceptible tomato cv. (as well as *V.albo-atrum* and eggplant), (Benhamou *et al.*, 1989). Other stress-induced molecules including **chitinases**, pathogenesis related-(**PR**) proteins (Benhamou *et al.*, 1990a) and hydroxyproline-rich glycoproteins (**HRGPs**) (Benhamou *et al.*, 1990b) are also important determinants of resistance expression. All the above plant reactions, usually occur even in the susceptible host but in a different time and space.

2. Mechanisms that deter or prevent the distribution of the pathogen within the vascular elements, secondary determinative phase (Beckman, 1989). Beckman and co-workers in a number of studies involving both *Verticillium* and *Fusarium* and different cultivars of tomato, cotton and banana studied the responses of the vascular elements after direct introduction of the pathogens into the vascular system, in addition to the introduction of vinyl-spore-size tracers particles that were used to trace the spores in primary infected xylem elements and to distinguish them from secondary infected xylem elements. By this system they obtained i) the pathogen in the xylem vessel, (where the pathogen is naturally found during the life of the host plants), ii) a uniform distribution of the spores into the xylem and iii) an easier way of localizing the pathogen. The same system of direct introduction of the parasite was also used by Robb and co-workers (Robb *et al.*, 1979a,1979b; Street *et al.*, 1986) using *V.albo-atrum*, *V.dahliae* and tomato and alfalfa cultivars as well as by Cooper and co-workers (Bishop & Cooper, 1984). In all studies, resistance was associated with the effective localization of the parasite by the plant and this appeared to involve the occlusion of infected vessels by the formation of **gels** or **gums** or **wall coatings** in general, **tyloses**, and the rapid **response of living xylem parenchyma contact cells** (formation of apposition layers, cytoplasmic responses, vacuolation).

Beckman *et al.* (1961) were the first researchers to introduce this system of investigation (spore infusion) to study vascular responses to wilt pathogens, showing that physical barriers such as perforation rims, verticulate lateral walls and gridded vessel endings function as trapping sites that delay further passage of spores (2-3 days were required for spores of the parasite to germinate and penetrate them), and provide time for host plant responses to operate and to successfully localize (or not) the infection. In a later

paper, Beckman *et al.* (1962) demonstrated 3 types of physical barriers: (a) perforation plates and vessel endings functioning as **trapping sites**, (b) **gel** formation produced beyond the initial barrier on the second day after inoculation, which temporarily immobilizes the spores and cuts off the water flow and (c) **tyloses**, that appear the 2-3d day after inoculation, that permanently seal off the invading vessel. In resistant cultivars of banana, gel formation was more persistent and extensive, and tyloses completely occluded the vessels by the 4th day after inoculation, at all temperatures tested; in the susceptible reaction, gels appeared in the first day but disappeared soon, allowing initial spore passage, and in addition there was considerable delay in the formation of tyloses at 27^0C. These resistance mechanisms did not function at all at 21^0C but provide a good resistance at $34\ ^0$C.

Gels were demonstrated to originate from pre-existing wall material by metabolic changes in the host, leading to plastidization and swelling of the primary cell wall membranes of perforation plates, end-walls within the vessels, and pit pairs between adjacent vessels, or pits between vessels and contact cells (VanderMolen *et al.*, 1977; Beckman & Talboys, 1981). This was shown to be a general response phenomenon, occurring after infection of nine different species of plants inoculated with *V.dahliae* and non-host-specific forma speciales of *Fusarium oxysporum* (VanderMolen *et al.*, 1977).

Tyloses appear 2-3 days after inoculation, developing as a result of parenchyma cells that adjoin the vessels (contact cells) producing outgrowths through the pits in vessel walls, thus completely walling-off the infected vessel above the trapping site. If the pits are too small to allow the passage of the growing cell into the vessel then the vascular elements are crashed. (Beckman, 1987). The walls of the tyloses become infused with stress metabolites (phenolics, phytoalexins), becoming lignified thus forming a 'lignified structure' and therefore are highly resistant to physical and chemical degradation (Beckman, 1966). Bishop & Cooper (1984) in an ulrastructural study of vascular colonization of resistant cultivars of tomato and pea infected with *F. ox. lycopersici*, *V. albo-atrum* and *F. oxysporum f.sp. pisi* respectively, found that both host species restricted the pathogens to the vascular tissue of roots and lower stems; in tomato, this occurred by occlusion of vessels with tyloses which was accompanied by the accumulation of an e-opaque material in the vacuoles of xylem parenchyma cells and in pea by gels. Tyloses were formed by increased vacuolation of xylem parenchyma cells (fusion of small vesicles originated from swollen rough ER), followed by deposition of a protective layer that became the tyloses wall. Gel formation arose from perforation plates, inter-tracheary pit membranes and was also extruded from xylem parenchyma pit membranes. This was pectinaceous in nature with cellulose and lignin, like that of vessel walls. Gel formation was also observed in the paramural space, suggesting there can be *de novo* synthesis of this material. VanderMolen *et al.* (1987) studied the ultrastructure of tyloses in resistant banana cv. following inoculation with *F. ox. cubensis*. Contact cells adjacent to vascular infection (8-48h after inoculation) were reacting by an additional apposition of a protective wall layer, while those that were some distance removed from the infection (2-8 days after inoculation) formed tyloses by plastidization of cell wall materials of pit membranes; tyloses walls appeared to arise from new Golgi-derived material. Tyloses were reported to be induced in tomato plants by IAA and ethylene (Pegg, 1959,1976). Beckman *et al.* (1982) found **callose appositions** in the walls of paravascular (contact) parenchyma cells of tomato cultivars (susceptible and resistant near isoline with the single dominant *I* gene which provides resistance to *F.ox. lycopersici*, race 1) after infection with *F. oxysporum* or root microflora, and this was shown to be a non-

specific response although the callose was deposited more rapidly in the Fusarium-resistant cv, than in the susceptible-isoline.

Robb *et al.* (1979a, 1979b) working with *V.dahliae* and chrysanthemum and sunflower as hosts also described **tyloses** as a response to infection and also 4 types of vessel **coating material**; smooth coating (type 1), fibrillar coating (type 2), bubbly coating (type 3) and irregular coating (type 4). Tyloses were found to contain carbohydrates and phenolic compounds; the fibrillar coating was found to have phenolics and no carbohydrates and the intensive Sudan black B response of both smooth and bubbly coatings indicated that they had a high lipid content (Robb *et al.*, 1979b). In a later paper by the same group (Street *et al.*, 1986), they showed that when resistant and susceptible tomato plants were infused with conidia of *V.albo-atrum*, secretion of wall-coating components was initiated. By the application of L-a-aminooxy-ß-phenylopropionate, a specific inhibitor of phenylopropanoid synthesis (a competitive inhibitor of phenylalanine ammonia lyase, PAL) resistant plants were converted to susceptible. This evidence showed that coating is important as a host defence mechanism and supports the theory that is secreted by the host plant as also reported by Moreau *et al.* (1973). Bishop & Cooper (1983b) studied the colonization of a susceptible cv. of tomato infected with *F.ox.lycopersici* and *V.albo-atrum*, and pea after infection with *F. ox. pisi*, and also described coating materials with smooth, rough, bubbly and less frequently fibrillar types that occluded inter-tracheary and xylem parenchyma pit cavities. Extensive erosion, and penetration of vessel walls was observed and death of xylem parenchyma (contact) cells was apparent following extensive symptom expression.

Efficient **coating** responses were found to be important for the defence mechanisms of alfalfa/lucerne plants to infection with *V.albo-atrum* by Robb and co-workers. In a susceptible cv, the suberin-like coating material was initiated at 8-12h post-inoculation in the ring of vessels surrounding the vessel containing the initial trapping site, but was delayed at the trapping site itself; on the contrary in the resistant cv. secretion began simultaneously (8-10h) in the vessels containing the trapping site and in contiguous vessels so forming barriers that prevented lateral invasion by the pathogen. Susceptible plants exhibited an extensive secondary colonization (Newcombe & Robb, 1988). Coating materials were found to be resistant to biodegradation by fungal exo-enzymes i.e. extracellular cell wall degrading enzymes [CWDEs], (Robb & Street, 1984). The different properties of coating material (chemical, physical, histochemical) indicated that they consist of lignin, or suberin, or both (Newcombe & Robb, 1988). In 1991, Robb *et al.* showed by direct chemical evidence that vascular coatings, formed by tomato plants in response to *V.albo-atrum*, or to a stress-response hormone, abscisic acid (that induces suberinization), mainly consisted of **suberin**. Phenylalanine ammonia lyase (**PAL**) is a plant defence enzyme which catalyses the deamination of I-phenylalanine to cinnamic acid, the first committed step to phenylopropanoid synthesis and to one of its products viz. suberin. Genetic analyses indicated that the enzyme (PAL) is encoded by a gene family, and in tomato, 5 distinct complementary gene loci were found (Lee *et al.*, 1992b). In the PAL 5 gene, two transcription initiation sites were found; when tomato plants were challenged by three different environmental stresses: wounding, light and fungal infection (*V.albo-atrum*), this gene was found to utilize the alternate initiation sites in a tissue- and stimulus-dependent manner (Lee *et al.*, 1994).

Contact cells appear to play an important role in defence mechanisms in vascular wilt diseases. These living xylem parenchyma cells that lie immediately adjacent to non-living vessels, in tomato have a distinctive wall apposition which contains a low concentration of pectin, a high concentration of polysaccharides and no lignin (Mueller & Beckman, 1984). One of the earliest responses of tomato infection was found to be the apposition within such contact cells of additional wall material with a marbled appearance which could be distinguished from protective layers because of its characteristic positive histochemical staining for **callose** (Mueller & Beckman, 1988). These callose deposits became visible 6-8h after inoculation and were infused with secondary metabolites that made them highly resistant to fungal infection. Susceptible and resistant cultivars of tomato both had the same capacity to respond to invasion to *F.ox. lycopersici*, but they differed in their degree of effective responses in the secondary xylem tissues; callose -containing wall appositions and HR responses were considerably more obvious in resistant cultivar of tomato, as also were the number of invaded contact cells (1st and 2d adjacent cells) (Beckman *et al.*, 1989). In another system with resistant and susceptible pea cvs to *F.ox.pisi*, contact cells of both cvs reacted in the same way to infection with the pathogen up to 4 days after inoculation by increase of cytoplasm and a decrease in the size of vacuoles, and the apposition of an e-dense deposit between the plasmalemma and the cell wall; also some of the contact cells were found to be necrotic. At the 4th day after inoculation, contact cells around initially infected vessels but which lacked the fungus in the susceptible cultivar remained highly cytoplasmic, while those contact cells in secondary infected vessels showed signs of disorganization, and by the 6th day these cells contained the fungus (Tessier *et al.*, 1990). Contact cells of resistant cultivars of tomato showed an early cytoplasmic response (60-90 min after inoculation) to *F.ox. lycopersici* at the sites proximal to the inoculum (Beckman *et al.*, 1991). Different types of carnation contact cells (viz. c3 , c4, c5, c6) appeared to be related to defence when injured or infected with non-pathogenic *V. dahliae* and with the specific vascular pathogen *Phialophora cinerescens.* Types c3 and c4 with large vacuoles, dense cytoplasm, well-developed ER, dictyosomes with high Golgi activity, accumulation of secretory products respectively appeared more frequently, and earlier, in plants inoculated with *V.dahliae*, whereas contact cells type c5 and c6 were more or less morbid and were present only after infection, not after wounding (c6 only in the compatible interaction) (Moreau & Catesson, 1985). Contact cells of initially- and secondarily-infected vessels of cotton reacted vigorously to infection to *V.dahliae* by the appearance of osmiophilic droplets that were secreted directly or indirectly into the vessel lumen; an apposition wall layer only appeared in the initially infected vessels (Mueller & Morgham, 1993).

Hormonal substances such as indole-acetic acid (IAA), ethylene and abscisic acid (ABA) have been shown to accumulate in plants with vascular infections. Ethylene is a very important factor that was found to be increased only in susceptible tomato plants infected with *V.albo-atrum* and also induced gelation in tomato plants (VanderMolen *et al.*, 1983).

Vascular browning is the common symptom observed in plants that have been infected with vascular wilt pathogens caused by dark pigments that occlude the vessels and block fungal, water and solute movement. This results from oxidised phenols that form quinones, which subsequently by oxidation and polymerisation with polysaccharides of pit membranes and adjacent parenchyma cell walls form pigments that accumulate in vessel lumen. The quinones interact with tryptophan that converts to

IAA and also inhibit the destructive oxidation of IAA, thus permitting its build-up. Phenol oxidizing enzymes as well as phenols are increased in infected tissues with all host species and races of wilt pathogens. Following vascular infection, phenolic glycosides that are located in specific cells (Mace, 1963) diffuse out of their cellular compartments (decompartmentalization) to vascular elements where they became hydrolyzed to free phenolics, then oxidized and gradually polymerized with other host elements becoming incorporated in lignified structures.

1.6. Metabolites produced by the pathogen

Vascular wilt pathogens produce a number of extracellular cellulolytic and pectolytic enzymes capable of attacking plant cell wall components. These enzymes have attracted much attention especially for the importance of the pectic fractions released from the plant cell walls in elicitation of defence reactions in the host. *V.dahliae* has been reported to produce pectin esterase, polygalacturonase (PG), pectate lyase (PL), glucosidase and cellulase (Cx), (Pegg, 1985). Pectolytic enzymes and especially endo-polygalacturonase, have been studied thoroughly in their relationship to vascular wilt symptoms and controversial results have been obtained; in some cases, symptoms were shown to be correlated with PG activity and virulence of the fungus, but in other cases, no such correlation was found. This was partially due to the different approaches used to study the contribution of these enzyme to disease, the diverse genetic backgrounds, the small number of individuals tested, and that multiple isoenzymes are involved. Heale & Gupta (1972) isolated exo-PL, PG and Cx from *V.albo-atrum*-infected lucerne; they found PL was coincident with symptom development and was produced before PG and Cx, PG being associated with the death of the host. Puhalla & Howell (1975) showed that *V.dahliae* endo-PG deficient mutants obtained from 3 defoliating isolates from cotton induced the same disease symptom to cotton as their parent strains. Tomato plants showed symptoms such as gels, vascular browning, wilting, chlorosis, necrosis and desiccation when treated with partially-purified PG and PL (Cooper & Wood, 1975). The same hydrolytic enzymes were found to increase in *V.dahliae*- inoculated oilseed rape plants, in the parts of plants showing typical symptoms of Verticillium after flowering, at the beginning of senescence; at the early plant growth stages their activities were undetectable, although activities of cellulase and xylanase were found (Dr. Holtschulte, personal communication).

Peroxidases and polyphenol oxidases can be produced both by the host and the pathogen . *In vitro* experiments with *V.dahliae* of potato (Lee & Tourneau, 1958) showed that chlorogenic acid is metabolized by the pathogen and can serve as a carbon source for the organism, but was also found to be inhibitor of fungal growth.

1.7. Wilt symptoms

A typical symptom (not always observed) of vascular diseases is true wilting or loss of turgor in the shoots. The petioles and leaves of affected plants droop because they do not receive enough water for maintenance of turgor. There is much controversy in the literature among plant pathologists who deal with vascular wilt diseases regarding the precise mechanisms causing wilting when the plant has been infected

with a vascular pathogen. The original explanation was that plants became blocked by the physical blockage of the vessels by the pathogen, thus causing a water shortage. The other explanation was that the pathogen produces a toxin or toxins that affect/s the plasma membrane function and cause/s water stress and this evidence was mainly supported by assays performed in plant cuttings when placed in solutions with the putative toxins obtained from culture filtrates. Verticillium has been reported to produce both low and high molecular weight phytotoxic metabolites in culture. Nachmias *et al.* (1982) showed that *V.dahliae* produces extracellular high molecular weight protein-lipopolysaccharide complexes (PLPC) that induce symptoms of Verticillium wilt. They provide evidence of the PLPC production *in vivo* by showing that an antigen that has been isolated only from infected *V.dahliae*-tomato plants was reacting with an antiserum prepared against PLPC. A specific binding site in plasma membranes of cotton seedlings for the PLPC has been demonstrated (Meyer & Dubery, 1993). Meyer *et al.* (1994) purified the phytotoxic protein-lipopolysaccharide complex (PLPC) from 7-day-old culture filtrates of *V.dahliae* which resulted in wilting, necrosis and elicitation of PAL activity in ~~treated~~ cotton seedlings. This PLPC could be dissociated into 5 protein-containing components; polygalacturonase, cellulase and 1,3-ß-Glucanase activities were associated with 4 of the protein components.

1.8. Glucosinolates

1.8.1. General structure and physical properties

Glucosinolates (also called mustard oil glucosides) are a large group of sulphur-containing, secondary plant metabolites. Gadamer (1897 a,b) first proposed the structural formula for 2-propenyl glucosinolate (sinigrin) and p-hydroxybenzyl glucosinolate (sinalbin), based on their principal chemical and enzymatic decomposition products. In 1956, Ettlinger & Lundeen amended this general structure to that confirmed by X-Ray crystallographic studies (Waser & Watson, 1963) and this is accepted today. The general molecular structure of all glucosinolates contains a ß-thioglucose grouping, a sulphonated oxime moiety and an organic side chain **R** (Figure-1.5-). Glucosinolates differ, and are distinguished individually, mainly by the nature of this side chain R. This side chain R determines the chemical, physical and secondary properties of the particular glucosinolate; it can be **aliphatic** (alkyl, alkenyl, hydroxyalkenyl, w-methylthioalkyl), or **aromatic** (benzyl, substituted benzyl), or **heterocyclic** (indolyl).

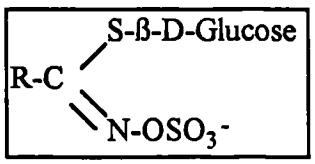


Figure -1.5- The general structure of glucosinolates.

Glucosinolates are all extremely hydrophilic, due to the sulphate and the thioglucoside components. The sulphate group also imparts non-volatile and acidic properties; the latter explains the presence of glucosinolates in nature as anions. All glucosinolates except sinigrin and sinalbin are difficult to crystallize, and for this reason have not been isolated and characterized as an intact structure. Their chemical structure has been deduced on the basis of their hydrolysis products (Underhill,1980).

1.8.2. Nomenclature

Initially, the nomenclature of glucosinolates was based on trivial names. The word 'gluco' was used as a prefix followed by the Latin name of the botanical species from which the glucosinolate had been first isolated, and suffixed with 'in', i.e.: gluco-nasturt-in from *Nasturtium officialis*. This system had very little functional value, since it did not indicate the anionic character of the compound, whereas the new semi-systematic system that Ettlinger and Dateo (1961) introduced uses names that derive from the chemical structure of the side chain R, followed by the word 'glucosinolate' (the suffix 'ate' at the end of the word: 'glucosinolate' indicates the anionic character of the compound). Thus, gluconasturtiin may be termed 2-phenylethyl glucosinolate. Table-1.1- gives the semi-systematic names of several glucosinolates found in oilseed rape, along with the trivial name, and the chemical structure of the aglucone side chain R.

Table-1.1-. Chemical structure, semi-systematic names and trivial names of aliphatic, aromatic (non-indole) and heterocyclic (indolyl) glucosinolates. All except sinigrin and glucotropaeolin are found in oilseed rape.

STRUCTURE OF R GROUP	SEMI-SYSTEMATIC NAMES	TRIVIAL NAMES
ALIPHATIC		
CH2=CH-CH2—	Allyl-glucosinolate	Sinigrin
CH2=CH-CH2-CH2-	But-3-enyl-glucosinolate	Gluconapin
CH2=CH-CH2-CH2-CH2-	Pent-4-enyl-glucosinolate	Glucobrassicanapin
CH2=CH-CHOH-CH2	(2R)-2-Hydroxybut-3-enyl-glucosinolate	Progoitrin
CH3-S-CH2-CH2-CHOH-CH2-	(2R)-2-Hydroxypent-4-eny-lglucosinolate	Napoleiferin
CH3-SO-CH2-CH2-CH2-CH2	4-Methylsulphinylbutyl-glucosinolate	Glucoraphanin
AROMATIC (non-indole)		
C6H5-CH2	Benzylglucosinolate	Glucotropaeolin
C6H5-CH2-CH2	Phenethylglucosinolate	Gluconasturtiin
HETEROCYCLIC (indole)		
C6H4-C5-CH2-NH	Indol-3-ylmethylglucosinolate	Glucobrassicin
C6H4-C3-CH2-NOCH3	N-Methoxyindol-3-ylmethylglucosinolate	Neoglucobrassicin
C6H3OH-C3-CH2-NH	4-Hydroxyindol-3-ylmethylglucosinolate	Hydroxyglucobrassicin
C6H3OCH3-C3-CH2-NH	4-Methoxyindol-3-ylmethylglucosinolate	Methoxyglucobrassicin

1.8.3. Occurrence

Glucosinolates are found extensively, but not exclusively, in the order Capparales, constituting the Capparaceae, Morigaceae, Resedaceae, Tovariaceae and Cruciferae (Kjaer, 1974). No authenticated member of the Cruciferae has yet been found to be devoid of glucosinolates (Fenwick *et al.*, 1983.). Other families in which glucosinolates occur occasionally are: the Limmanthaceae, Caricaceae, Tropaeolaceae, Gyrostemonaceae, Salvadoraceae and Euphorbiaceae (Kjaer, 1974). There is a wide variation in the distribution of glucosinolates among the 11 botanical families mentioned above. Methyl-glucosinolate (glucocapparin) rarely occurs outside the family Caparaceae, whereas benzyl glucosinolate has a broad family distribution (Underhill, 1980). Rodman (1981) in an in-depth review of the taxonomic distribution of glucosinolates, showed a positive correlation between species diversity within a family and the structural diversity of its glucosinolates, thus proposing its chemotaxonomical use. Daxenbichler *et al.* (1991) demonstrated the glucosinolate composition in seeds of 297 species of 9 families including the Capparidaceae, Cruciferae, Caricaceae, Morigaceae, Phytolacaceae, Pittosporaceae, Resedaceae, Salvadoraceae and Tropaeolaceae.

Approximately one hundred different glucosinolates have been characterized which differ in the molecular structure of the side-chain R. This side chain includes aliphatic, aromatic (no-indolic) and heteroaromatic (indolic) groups. The classification of glucosinolates in Table-1.1- is mainly based upon the structure of the side chain R.

Glucosinolates are not confined to any particular part of the plant, but occur in all tissues of an oilseed rape plant; their concentrations vary considerably according to age, declining during germination. Concentrations reach a maximum level in the seed where the endosperm is the main site of accumulation (Olsen & Sörensen, 1980). Certain glucosinolates found in the seed may not be present at all, or occur in trace amounts only in the developing and mature plant. There is also a remarkable variation in glucosinolate profiles from different tissues within one plant, when different Brassicas were examined (Sang *et al.*, 1984). When individual glucosinolates from different parts of an oilseed rape seedling were measured and expressed on the basis of the whole plant, the glucosinolate profiles varied independently during development, and were related to the development of the specific organ and tissue from which they had been extracted (MacGregor, 1988). Variation was also observed in both the proportion and concentration of individual glucosinolates in the stems and leaves of 3 different cvs of *Brassica napus* L. (MacfarlaneSmith & Griffiths, 1988). Additionally, the total and individual glucosinolate content depend upon the variety, cultivation conditions, climate and agronomic practices. Both the 'single' and 'double' low varieties of rape showed the same pattern of indolyl-glucosinolates (Uppström,1983). 'Double low' glucosinolate varieties differ from 'single low' glucosinolate varieties only in the amount of aliphatic glucosinolates which has been shown to be specific to the seed (Milford *et al.*, 1989; Inglis *et al.*, 1992). There is no obvious relationship between concentration of total glucosinolates in the seed and that in plants grown from them (Baig, 1991). Thus, glucosinolate levels in the seed do not reflect the glucosinolate levels in the vegetative parts of the plant except in the very young seedlings and cotyledon stage (Uppström, 1983; Glen *et al.*, 1990). The 'double-low' varieties at this young stage show a very low level of glucosinolates throughout all parts of the plant, reflecting the amount present in the seed, but 'single-low' varieties start with a higher content, which decreases during the development of the seedling and cotyledon (Uppström, 1983).

All the above evidence indicate that total and individual glucosinolate concentration in the seed varies greatly depending upon the variety, cultivation, climate and stresses, so that glucosinolates in the vegetative part of the plant cannot be accurately determined from predictions based on the glucosinolate content in the parental seed from which they developed from. Furthermore, their profiles also depend on various parameters such as variety, type of tissue, developmental stage and growth conditions.

1.8.4. Biosynthesis of glucosinolates

Several biosynthetic studies, involving feeding experiments with synthesized radiolabelled compounds, isolation of intermediates and detection of some enzymes involving in the pathway, have revealed that glucosinolate side chains have derived from both protein, and non-protein, amino acids. Subsequently, the glucone moiety is formed through a complex series of several nitrogenous, and sulphur-containing, intermediates. Aliphatic glucosinolates like glucocapparin (methylglucosinolate) are formed from alanine. Aliphatic glucosinolates with chain length >C3 are derived from methionine (Underhill,1980), and have to undergo a chain elongation mechanism before glucosinolate biosynthesis. Benzyl and phenethyl glucosinolates derive from phenylalanine, and the indole glucosinolates derive from tryptophan. From a variety of studies, it has been proposed that all glucosinolates are formed by a common biosynthetic

pathway (Underhill, 1980). The aromatic amino acids required for aromatic glucosinolate formation (phenylalanine and tryptophan) are produced via the shikimic acid pathway; this pathway is also very important for the production of lignins, aromatic amino acids and phenolic compounds.

The biosynthetic pathway illustrated in Figure-1.6- is based on Underhill (1980) and involves 5 main steps. The first step involves the formation of N-hydroxyaminoacid [catalysed by glyoxylate aminotransferase (MGAT)], which is decarboxylated to give aldoxine (second step). Aldoximes are not only involved in the formation of glucosinolates, but are important intermediates in the biosynthesis of cyanogenic glucosides, nitriles, amides, aldehydes and alcohols (Mahadevan, 1973). It has been suggested that the aldoxime may undergo oxidation to a primary nitro compound, the aci-tautomer of which is the locus of the introduction of the sulphide-S. Feeding experiments have shown that cystein is the S-donor, thus producing thiohydroximate (third -step). The precise mechanism of sulphur insertion, and which enzymes are involved, is as yet uncertain. The thiohydroximate is glycosylated by a glycosyl transfer from UDP-glucose producing the desulphoglucosinolate (step 4); the final step involves the sulphation of desulphoglucosinolate, mediated by a sulphotransferase, which catalyses the transfer of sulphate from 3-phosphoroadenosine-5'-phosphorosulphate (PAPS) to desulphoglucosinolate. The addition of the hydroxy group to alkenyl glucosinolates (gluconapin, glucobrassicanapin) to produce the hydroxyalkenyl analogues (progoitrin, napoleiferin), occurs after the formation of the glucosinolate molecules (Underhill & Wetter., 1973).

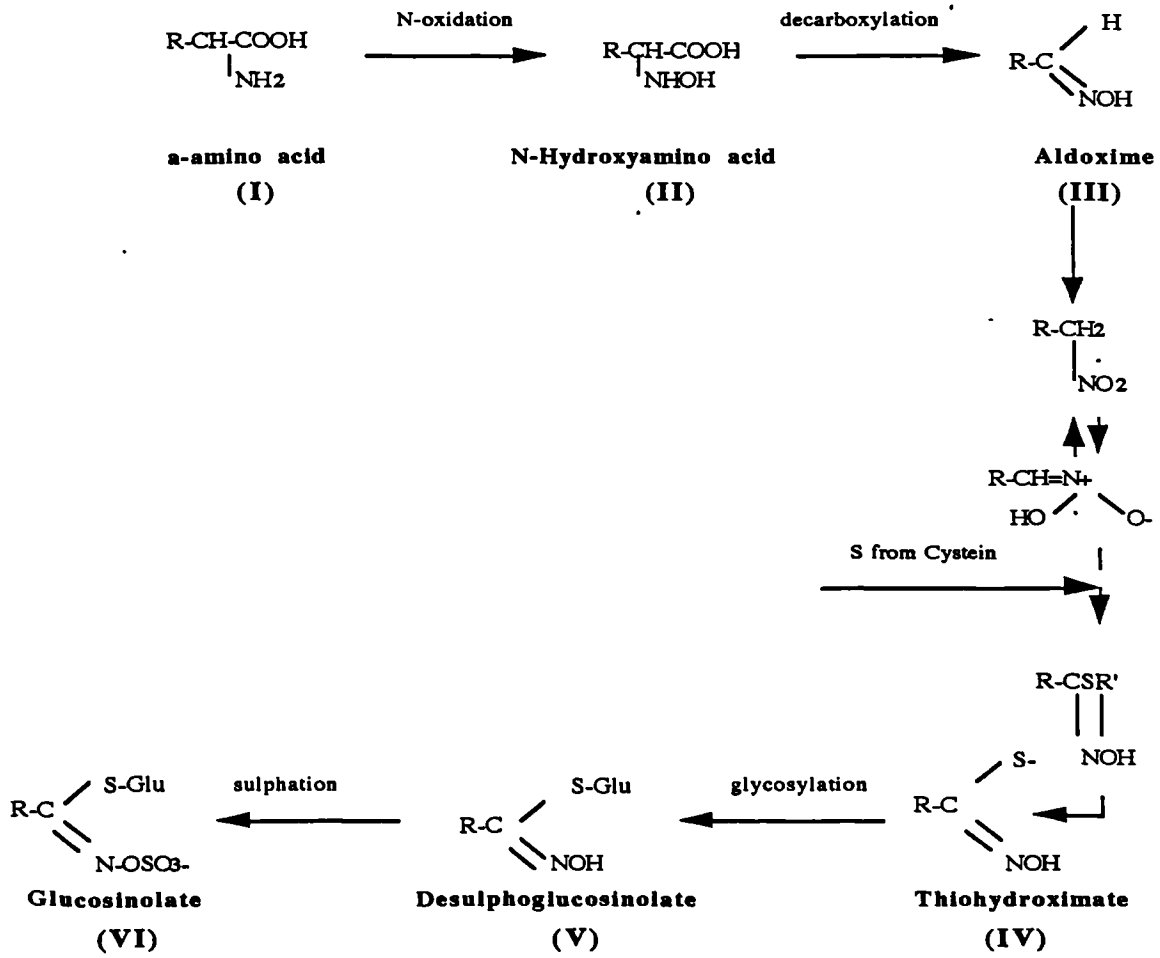


Figure-1.6- The biosynthesis of glucosinolates (Based on Underhill, 1980).

1.8.5. Enzymatic degradation of glucosinolates

Glucosinolates are hydrolysed by the endogenous enzyme, myrosinase, and by an exogenous enzyme, sulphatase.

1.8.5.1. Sulphatase

Sulphatase (aryl-sulphate sulphohydrolase, EC 3.1.6.1) from the edible snail (*Helix pomatia)* is responsible for the hydrolysis of glucosinolates to desulphoglucosinolates (see Figure-1.7-). This reaction was used in this project to convert glucosinolates to desulphoglucosinolates which were subsequently analysed by HPLC.

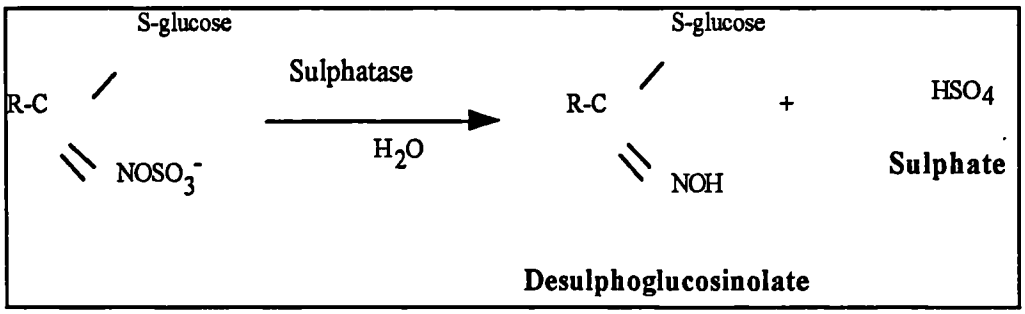


Figure-1.7- Hydrolysis of glucosinolates to desulphoglucosinolates.

1. 8. 5. 2. Myrosinase

Myrosinase is the trivial name for the endogenous group of enzymes (ß-thioglucosidase, thioglucoside glycohydrolase, E.C.3.2.3.1) responsible for the hydrolytic cleavage of the thioglucoside bond of glucosinolates, forming an unstable **aglucone** and **glucose** (see Figure -1.8-, below).

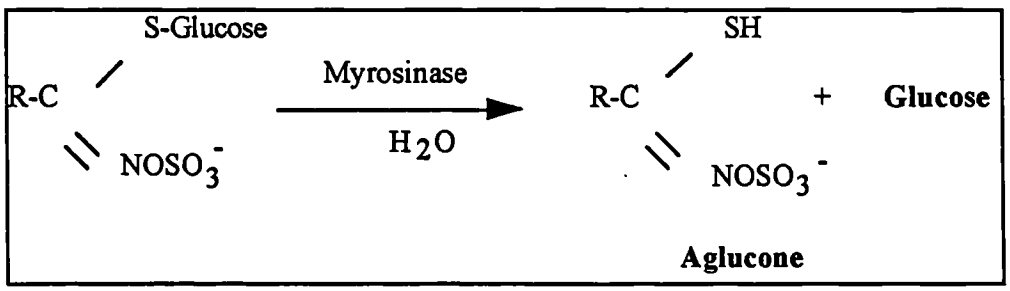


Figure-1.8.- Hydrolysis of glucosinolates to glucose and aglucone by myrosinase.

This enzyme was first reported by Bussy (1840), who investigated mustard oil glucosides in *Brassica nigra* L., and described a substance with albumin properties which he called myosyn, that was responsible for the hydrolysis of sinigrin.

Earliest theories suggested that the enzyme was both a glucosidase that cleaves the thioglucosidic linkage, and a sulphatase, that cleaves the sulphuric ester linkage. Euler & Erikson (1926) and Neuberg & Schoenbeck (1933) claimed the separation of these two moieties. Today, it is considered that myrosinase behaves only as thioglucosidase, producing **glucose** and **aglucone** products (thiohydroximate-D-sulphonates), the former being unstable, forming a variety of products depending on:

(i) the chemical structure of the side chain R,

(ii) the concentration of the H^+ ions,

(iii) temperature,

(iv) presence of metal ions,

(v) concentration of protein co-factors (ESP, epithiospecifier protein) MacLeod & Rossiter (1987).

Glucosinolates with an alkyl, alkenyl or aromatic side chain (the majority of glucosinolates) form mainly isothiocyanates (at pH 5-7), or nitriles (at pH 3), thiocyanates and epithionitriles (see Figure-1.9-).

Glucosinolates with ß or g hydroxylated alkenyl side chain ie: progoitrin, napoleiferin, form unstable hydroxyisothiocyanates which cyclise to oxazolidine-2-thiones. At low pH, hydroxynitriles are produced. In the presence of metal ions and ESP (epithiospecifier protein), epithionitriles are produced (Figure-.1.9-).

Glucosinolates with indole side chains ie: glucobrassicin, neoglucobrassicin, methoxyglucobrassicin, hydroxyglucobrassicin, form unstable isothiocyanates at pH 4-7 which degrade to produce indolyl alcohols (indolyl-3-carbinol) and the thiocyanate ion. The alcohol may condense to form diindolylmethane or react with ascorbic acid to form ascorbigen. It is possible that the unstable isothiocyanates may be precursors to indole phytoalexins that are induced in Brassica species, following biotic and abiotic elicitation (Rouxel *et al.*, 1989). At pH 4, indolylacetonitrile is produced (see Figure-1.10-).

Thus, the major degradation products are **isothiocyanates** and **oxazolidine-2 thiones.** At neutral or high pH, isothiocyanates (known as mustard oils) and **sulphate** molecules are produced by a 'Lossen rearrangement' (migration of the nitrogen) as postulated by Ettlinger & Lundeen (1956). At low pH, **nitriles** and elemental **sulphur** are produced. The presence of ferrous ion also favours production of nitrile, rather than isothiocyanate or oxazolidine-2 thione. At low pH, indolyl glucosinolate (3-indolylmethyl) forms 3-indolylacetonitrile (IAA) which is a growth -promoting compound; in addition, organic **thiocyanates** rather than isothiocyanates are produced, but the reasons for this at the present are unclear (Underhill,1980).

Glucosinolates with terminally unsaturated carbon atoms in the presence of ferrous ion and ESP, form **epithionitriles.** ESP was first isolated from *Crambe abyssinica* seeds (Tookey, 1973). It is a small protein (30-40kDa), with no thioglucosidase activity, which interacts with myrosinase and ferrous ions to transfer sulphur from the S-glucose moiety to the terminal double bond specifically, forming epithionitriles (episulphides).

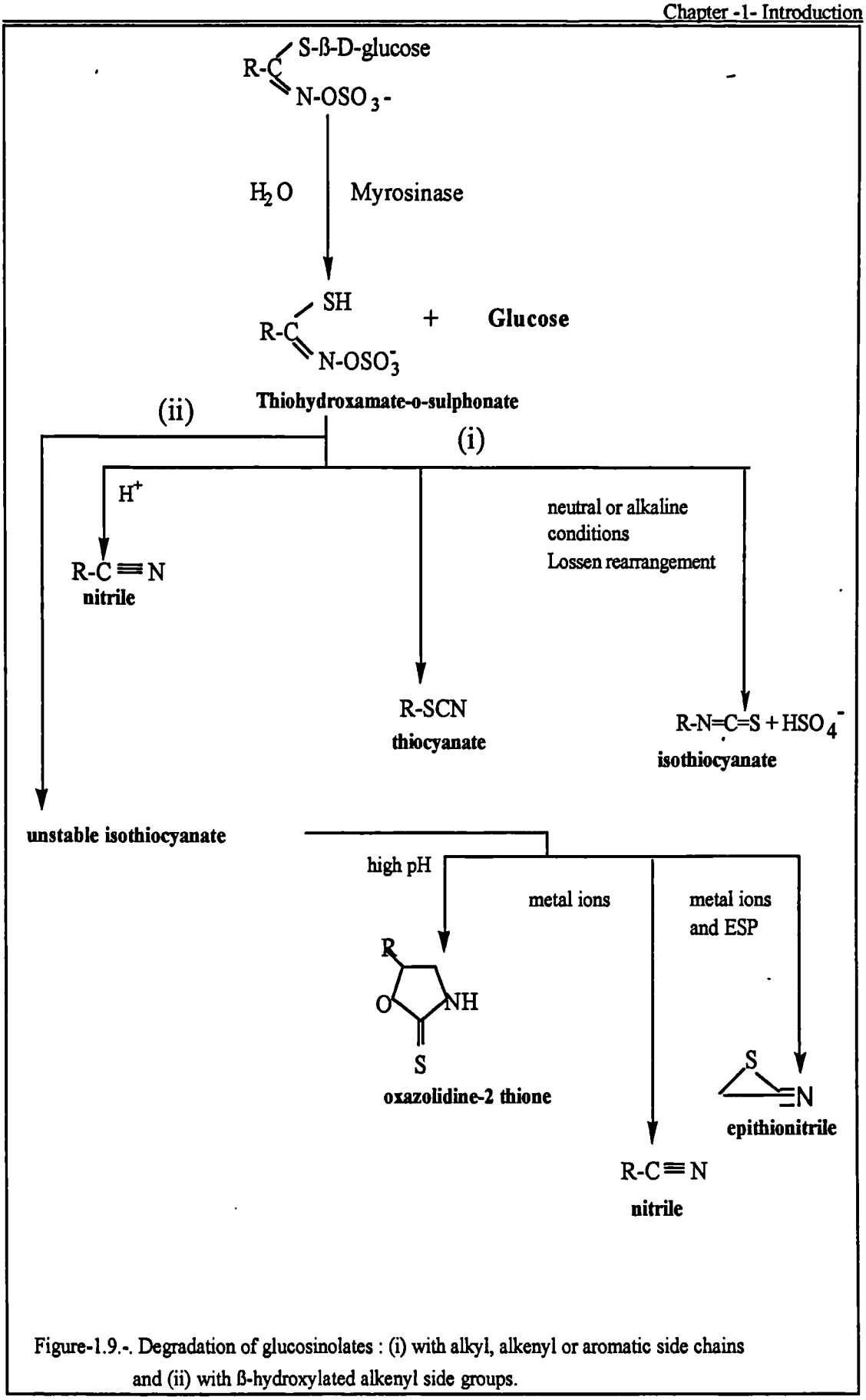


Figure-1.9.-. Degradation of glucosinolates : (i) with alkyl, alkenyl or aromatic side chains and (ii) with ß-hydroxylated alkenyl side groups.

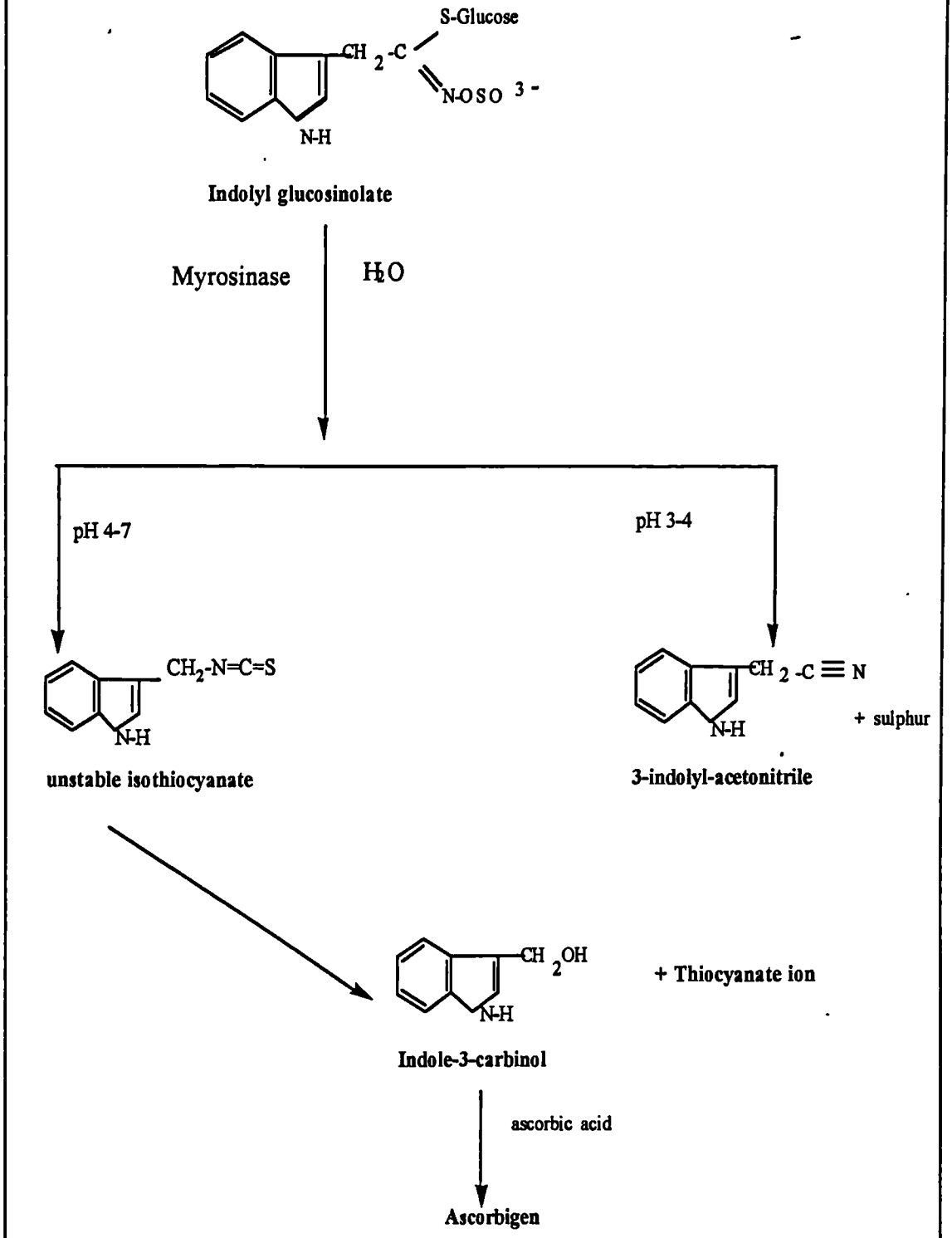


Figure-1.10-. Degradation of glucosinolates :(iii) 3-indolyl-methyl side chain.

1.8.6. Biological properties of glucosinolates and their hydrolysis products.

A number of studies have been shown that glucosinolates and their hydrolysis products have beneficial and undesirable effects. They are responsible for:

(i) The flavour of vegetables. The pungent taste of mustard is caused by the enzymatic hydrolysis of allylglucosinolate (propenyl) to allylisothiocyanate (2-propenyl-isothiocyanate). The natural flavour of cabbage is mainly attributed to 2-propenyl isothiocyanate, the hydrolysis product of propenylglucosinolate. This glucosinolate, along with oxazolidine-2-thione (VOT), are also responsible for the bitterness in Brussels sprout, cabbage and swede. Additionally, the hotness of horseradish has been ascribed to 2-propenyl and phenylethyl isothiocyanates. The reason why frozen Brussels sprouts do not have the same flavour as the freshly cooked ones, is because of the low concentration of isothiocyanates due to inactivation of myrosinase by freezing (MacLeod, 1976). Generally, isothiocyanates, in a relatively low concentration have a pleasant, appetite-stimulating flavour, whereas nitriles have a garlic-like flavour (Underhill,1980).

(ii) Toxicity to vertebrates. Investigations of physiological effects on animals of glucosinolates and/or their breakdown products, using isolated intact glucosinolate and active myrosinase in animal diets or rapeseed meals with known glucosinolate content, reveal their toxicity and their antinutritional properties. Glucosinolate breakdown products such as VOT and isothiocyanate ion have goitrogenic effects on animals fed with meal containing high levels of glucosinolates, but these effects have not been detected in humans. Nitriles (hydroxynitriles, epithionitriles) are also the most toxic breakdown products of glucosinolates, and are hepatotoxic (Heaney & Fenwick, 1987).

(iii) Toxicity to microbes and disease resistance. Isothiocyanates , the major glucosinolate breakdown products are volatile, and possess wide antifungal and antibacterial properties.

Greenhalgh & Mitchell, (1976) demonstrated the toxic effect of 2-propenyl-isothiocyanate, the volatile breakdown product of sinigrin, on sporangial germination of *Peronospora parasitica*, and showed that the one cultivar that was resistant to the pathogen yielded the greatest amounts of this volatile after tissue maceration, suggesting that the resistance of certain Brassicas to cabbage downy mildew was due to the release of this chemical compound. Peterka & Schlosser (1989) showed the inhibitory effects of the breakdown products (isothiocyanates) of sinigrin, gluconapin, and glucobrassicanapin on the growth of the pathogen *Leptosphaeria maculans* (stem canker). They also demonstrated the correlation between sinigrin and gluconasturtiin content in cotyledons in 2 varieties of *B. napus* and one variety of *B.juncea* and resistance to this pathogen; *B. napus* varieties which lacked sinigrin, and with lower levels of gluconasturtiin in their cotyledons, showed reduced resistance to the pathogen as compared with the *B. juncea* variety. Mithen *et al.*, (1986) investigated the biological effects of 5 glucosinolates (sinigrin, gluconapin, progoitrin, glucobrassicin and methoxyglucobrassicin), and their hydrolysis breakdown products, on growth of *Leptosphaeria maculans*, and found that all glucosinolates except progoitrin, when added to the culture media containing myrosinase, reduced fungal growth, and that sinigrin hydrolysis

products at pH:7 (presumably allyl-isothiocyanate) had the more pronounced effects. In the same investigation, the authors showed the inhibitory effect of glucobrassicin breakdown products on *L.maculans* growth, and indol-3-carbinol proved to have the greatest antifungal activity. Allyl-isothiocyanate was also found to be toxic to *Nematospora sinecauda* (yeast seed pathogen) at concentrations well within the range expected in seeds (Holley & Jones, 1985).

Mari *et al.* (1993) in a study on the antifungal properties of isothiocyanates formed by 6 glucosinolates in natural pH (pH: 7) to post-harvest fruit pathogens: *Botrytis cinerea*, *Rhizopus stolonifer*, *Monilia laxa*, *Mucor piriformis* and *Penicillium expansum*, showed that the isothiocyanates exhibited antifungal activities with different specificities. Those derived from glucoraphanin, sinigrin and sinalbin completely inhibited conidial germination of all 5 pathogens tested, and sinigrin-derived isothiocyanate (allyl-isothiocyanate) was the more active, which is consistent with the above findings. Mithen *et al.* (1987) showed that Brassica species with higher levels of alkenyl glucosinolates in their leaves, were more resistant to *L. maculans* (causing small localized lesions) as compared with Brassica susceptible lines (systemic infection, large lesions). However, the same study showed that the hypersensitive response was not correlated with glucosinolate levels.

Spore germination and growth of *V.dahliae* and *V.d.longisporum* isolates were shown to be strongly inhibited in liquid cultures in the presence of glucosinolates (except of progoitrin) and myrosinase; the higher levels of inhibition were exhibited by the gluconasturtiin breakdown products at pH: 7 viz. phenylethyl isothiocyanate (Baig, 1991). The inhibitory effects of volatile compounds from glucosinolates to mycelial growth and microsclerotia formation in *V. dahliae* has also been demonstrated recently by Melouk *et al.* (1994) who used decomposed rapeseed meal with known concentrations of glucosinolates in the soil and examined the growth of the fungus in a time course study. In the same paper, they also showed that melanin production was also reduced in *V.dahliae* cultures exposed to volatiles derived from decomposed rapeseed meal. Buchwaldt *et al.* (1985) demonstrated the effect of sinigrin on pigmentation of *Alternaria brassicae* when this glucosinolate was added to the Czapek Dox growth medium, while the same glucosinolate had no, or only a weak, effect on *Phoma lingam* and *Sclerotinia sclerotiorum.*

(iv) Insect attraction and deterrence. Glucosinolate breakdown products (e.g. isothiocyanates) have been shown by several workers to influence (attract/repulse) the adults of different insect species to the host plant, while intact glucosinolates influence their behaviour and egg-laying when contact with the plant has been made (Chew, 1988). Huang *et al.* (1993) showed that the differential acceptance of *Erysimum cheiranthoides* by *Pieris rapae* and *Pieris napi oleracea* was partly due to stimulant effects of the two glucosinolates, glucocheirolin and glucoiberin, on *P.n.oleracea.*

1.8.7. Induction of glucosinolates

A number of studies have been shown that Brassica pests and pathogens, as well as damage (artificial wounding) all induce indole-glucosinolate synthesis, and in some cases, aliphatic glucosinolate synthesis.

The development of 'clubbing' caused by the fungus (also regarded as a slime mould) *Plasmodiophora brassicae*, is associated with a large increase in both glucobrassicin (an indole-glucosinolate) and its degradation product: indolylacetonitrile (Butcher *et al.*, 1974), which has been shown to be converted in turn to the growth hormone IAA (Searle *et al.*, 1982). Indolyl- and aralkyl-glucosinolates accumulated in the 6th leaf of both 'Bienvenu' and 'Cobra' cultivars of oilseed rape at 16 days-post inoculation with the fungus *Alternaria brassicae*, while aliphatic glucosinolates increased in the 6th leaf in only the 'single zero' cv. 'Bienvenu' at 9 days post-inoculation (Doughty *et al.* 1991). Birch *et al.* (1990) showed that the concentration of indole-glucosinolates (glucobrassicin and neoglucobrassicin) was increased in the roots of all rapes tested (3 forage rapes and 2 winter oilseed rapes) infested with *Delia floralis*, while the aliphatic glucosinolates decreased. The major increase was in the neoglucobrassicin concentration, which accounted for the majority of the observed compositional changes. The decrease 60% in the total aliphatic glucosinolates of infested roots was due to progoitrin. Later, Griffiths *et al.* (1994), in a study on the effect of *Delia floralis* larval damage on glucosinolates of one oilseed cv. 'Ariana' and two forage rape cvs: 'Bonar' and 'Hobson' of *Brassica napus* showed that larval damage increased the proportion of indole-glucosinolates in roots and stems, but had no effect on the glucosinolate content of leaves of plants whose roots had been damaged with the larvae of the pest. Neoglucobrassicin increased in larval-damaged roots, but artificial damage had no effect on neoglucobrassicin concentration in roots. Infestation of petioles, lamina and stems of *Brassica napus* with *Psilliodes chrysochephalla* led to an overall reduction of aliphatic glucosinolates (both alkenyl- and hydroxyalkenyl-), whereas the levels of the indole-glucosinolates (the major being glucobrassicin) increased. Punctured petioles and laminae showed the same induction, but the levels of the indole-glucosinolates were higher in the infested plant tissues (Koritsas *et al.*, 1989). Plants that had been infested in the field showed the same induction of indole glucosinolates, and reduction of aliphatic glucosinolates. Neoglucobrassicin was also increased in roots of plants infested with the cabbage stem flea beetle, while aliphatic- glucosinolates were decreased in laboratory studies (Koritsas *et al.*, 1991). In the same study, artificial damage to kale led to an increase in both aliphatic and indole-glucosinolates, while mustard plants (*B.nigra*, *B.juncea*, *S.alba*) showed an increase in the aliphatic glucosinolates: sinigrin (*B.nigra*, *B.juncea*) and sinalbin (*S.alba*).

Bodnaryk (1992) showed that wounding (artificial damage) in cotyledons of 4 different genotypes of Cruciferae (*B.napus*, *B.rapa*, *B.juncea* and *S.alba*) resulted in an increase of indole-glucosinolates (glucobrassicin, neoglucobrassicin) in all Brassica species studied; hydroxybenzyl glucosinolate, which is the predominant glucosinolate in *S.alba*, did not change in concentration after wounding. In the same study, the authors demonstrated the positive effect of cotyledon wounding to the concentration of glucobrassicin of the 1st and sister cotyledon. Plants that had been grown under darkness did not show so much increase in indole-glucosinolates as plants grown in the light, and sulphur-deficient plants showed greater percentage increases of indole-glucosinolates than plants growing with the sulphate ion. Table-1.2- gives a summary of glucosinolate induction/reduction found by different investigators after wounding, pest infestation and fungal infection.

Table-1.2.- Summary of changes in indole- and aliphatic-glucosinolates after pest infestation, fungal infection and wounding damage of tissues of Brassica plants.

CAUSE	INDOLYL-	ALIPHATIC-	TISSUE	REFERENCE
Plasmodiophora brassicae	Increase glucobrassicin		roots, stems	Butcher *et al.* 1974
Alternaria brassicae	Incr. Cobra Incr. Bienvenu	Decrease Cobra Incr. Bienvenu	leaves	Doughty *et al.* 1991
Delia floralis	Increase neo-/glucosbrassicin	Decrease	roots, stems, leaves	Birch *et al.* 1990, Griffiths *et al.* 1994
Psylliodes chrysocephalla	Increase neo-/glucobrassicin	Decrease	petioles, stem, lamina, roots	Koritsas *et al.* (1989, 1991)
Phyllotreta cruciferae	Increase glucobrassicin		cotyledons	Bodnaryk 1992
Wounding	Increase glucobrassicin		cotyledons	Bodnaryk 1992

Incr. = Increase

1.8.8. Localization of glucosinolates

Earlier work by different authors has shown that glucosinolates are localized in cell vacuoles. Grob & Matile (1979) isolated vacuoles from plasmolysed (*Armoracia lapathifolia*) horseradish root tissue, and by using phenolic compounds as vacuolar markers (equal recovery yield of phenolics and glucosinolates), they concluded that glucosinolates (sinigrin and gluconasturtiin) have a subcellular localization in the large central vacuoles of *Armoracia lapathifolia* (horse radish) root cells. They also detected myrosinase activity in vacuolar fractions. In 1992, Wei *et al.* localized glucosinolates in roots of *Sinapis alba* by X-ray microanalysis in dry-cut, cryosections of fresh frozen roots; sulphur, one of the main elements of glucosinolates was the marker. They found high concentrations of sulphur in the vacuoles of root cap cells, and also in the vacuoles of cortex cells at more than 0.2 mm from the root tip. The less differentiated cells with smaller vacuoles contained less sulphur=glucosinolate content. They also detected electron opaque polyphosphate granules in the vacuoles. Additionally Helmlinger *et al.* (1983), provided evidence for the vacuolar location of the indole-3-methyl-glucosinolate (glucobrassicin) and N-Methoxyindol-3-methyl-glucosinolate (neoglucobrassicin) in storage root tissues of *Armoracia rusticana* (horseradish) by feeding experiments with [^{14}C]-tryptophan and by using acid phosphatase as vacuolar marker. Later in 1984, Yiu *et al.*, using a staining marker: TCQ, (N,2,6-trichloro-p-benzoquinoneimine) detected glucosinolates within protein bodies in the cotyledonary cells of hand-cut sections of rapeseed (*Brassica campestris* L. cv. 'Candle'). The protein bodies stained bright yellow in the presence of glucosinolates.

1.9. Myrosinase

1.9.1. Occurrence of myrosinase

Myrosinase activity has been detected in all Brassicaceae plants containing one or more glucosinolates, and recently in a member of Cucurbitaceae (*Cucumis melo*) [Bois *et al.*,1992]. It is not only confined to plants. Several researchers have demonstrated myrosinase activity from other organisms: Ohtsuru &Hata (1973) in *Aspergillus niger;* Reese *et al.* (1958) in *A. sydowi*; Tani *et al.* (1974) in *Enterobacter clocae*; Oginsky *et al.* (1965) in strains of *Escerichia coli* and *Paracolobactrum aerogenoides* isolated from man; Nugon-Bandon *et al.* (1990) in intestinal lactobacillus (LEM 220); Goodman *et al* (1959) in mammalian tissue tumours; MacGibbon & Allison (1968) in *Brevicorynae brassicae* and MacGibbon & Beuzenberg (1978) in *Lipaphis erysimi.*

1.9.2. Isoenzymes

Several investigators have shown that there are multiple forms of myrosinase in large number of Cruciferae plants. Vaughan *et al.* (1968) demonstrated myrosinase isoenzymes by gel electrophoresis. MacGibbon & Allison (1970) isolated several isoforms of myrosinase from 7 different species of Rhoedales, including several tissues (leaves, seeds, roots, petioles), and they found each species to contain a distinct individual pattern of isoenzymes that depended on the type of tissue from which the enzyme had been extracted. Rapeseed (*Brassica napus* L.) has been found to contain at least 3 isoenzymes (all glycoproteins) as recorded by Lönnerdal & Janson (1973), with no significant variation in substrate specificity, according to Björkmann & Lönnerdal (1973). Buchwaldt *et al.* (1986), using fast protein liquid chromatography, found more than one isoenzyme in *Brassica napus*, *Brassica nigra* and *Sinapis alba*. Further, Bones & Slupphaug (1989) isolated and purified 3 isoforms of the enzyme from rapeseed. James & Rossiter (1991) separated 2 myrosinases from cotyledons of 5 day-old OSR seedlings. Myrosinase I (156kDa) was distributed throughout the seedling (roots, hypocotyls and cotyledons), and was shown to be glycosylated, whereas myrosinase II was located exclusively in cotyledons and found to be less glycosylated when analysed with periodic acid-Schiff stain. Myrosinase I was purified and polyclonal antibodies raised in rabbit against this protein. These polyclonal antibodies (M1) were used in the present study for the immunogold localization of myrosinase in *Brassica napus* cv. 'Cobra'.

1.9.3. Activation

Almost all the isoenzymes of myrosinase described are activated by L-ascorbic acid, but the extent of activation varies. Ettlinger *et al.* (1961) isolated two myrosinase isoenzymes from the same species (*Sinapis alba*), and found that one was activated and the other was not. Further, Henderson & Mc Ewen (1972) studied the effect of ascorbic acid on different isoenzymes from 3 different species (*B.napus*, *B.nigra* and *S.alba*) and found that there was variation in response to ascorbic acid among the different species examined, and also between equivalent isoenzymes. Wilkinson *et al.*(1984), studied the myrosinase

activity of 12 Cruciferous vegetables and concluded that the ascorbate concentration promoting maximum activity varied within the 6 species included. MacLeod & Rossiter (1987) demonstrated the activation of progoitrin by ascorbic acid (1.57mM) by a factor of 100, and also described the inhibitory effects of Fe^{+2} and Cu^{+2} on myrosinase activity. James & Rossiter (1991) reported that both myrosinase I and myrosinase II were activated by ascorbic acid, and that the maximum activation for myrosinase I was 50-fold at 0.3mM, and for myrosinase II was 106-fold at 0.3-0.5mM.

The myrosinase activity function of ascorbic acid was first reported by Nagashima & Uchiyama (1959), who showed that L-ascorbic acid does not behave as a conventional reducing agent, and that myrosinase was not activated by most reducing agents. Tsuruo & Hata (1968) presented a model for the activation and inhibition of myrosinase by ascorbic acid. They postulated that there were two binding sites for ascorbic acid; one next to the substrate site for the aglucone part of the glucosinolate, and the other at the substrate site for the glucone part of the glucosinolate. When ascorbic acid is in the reaction, it occupies the binding site next to the substrate site for the aglucone part of the glucosinolate, and alters its conformational site so that the aglucone part fits better to the substrate site, and the reaction is thus activated. When higher concentrations of ascorbic acid are involved in the reaction, the ascorbic acid binds in the substrate site for the glucone part of the glucosinolate, thus preventing the binding of glucosinolate, and inhibiting the reaction. When p-nitrophenyl-ß-glucoside (p-NGP) is used as a substrate, hydrolysis is not activated because p-NGP does not use the substrate site for the aglucone part. Ohtsuru & Hata (1979) have concluded that the ascorbic acid is not itself involved in the catalysis, but that it changes the conformation of the active site of the enzyme when the effector binding sites are occupied. Grob & Matile (1980) found that the concentrations of ascorbic acid of horse radish tissue were about 2mM, which is a value close to that found (1.8mM) for maximum myrosinase activity. In the presence of ascorbic acid, horse radish myrosinase was activated by a factor of 800-1000. The same group, using phenolic compounds and acid phosphatase as vacuolar markers, showed that ascorbate was localized in isolated cellular vacuoles in horse radish roots.

1.9.4. Localization of myrosinase

A number of studies have been performed during the last century to localize the enzyme or its site of activity in very young tissue or the developing embryo of Brassicaceae species, using a varieties of techniques (morphological, histochemical, cytochemical, cell fraction techniques, immunocytochemical). The enzyme was localized either in different organelles in the cytoplasm in unspecialized cells (especially when the site of myrosinase activity was related to the location of the enzyme), or in specific cells, idioplasts (myrosin cells) in myrosin grains.

1.9.4.1. Localization in unspecialized cells

To localize the enzyme *in situ* by EM, Iversen (1970) took advantage of the fact that an electron-dense precipitate of lead sulphate forms during the hydrolysis of glucosinolates at the localized sites of myrosinase activity. In a detailed study of different plant families, he demonstrated the occurrence and

structure of dilated cisternae of ER which showed accumulations of a fine granular or fibrillate nature, appearing to be unique to the Cruciferae, and were shown to be common feature of root, stem and leaves of these plants. Demonstration of the limited and specific occurrence of the organelles, as well as the enzyme myrosinase in members of Cruciferae suggested that the enzyme might be localized in the dilated cisternae. Incubation in formaldehyde-fixed root tips (3days+2days) of *Sinapis alba* resulted in precipitation of electron-opaque deposits on membranes of mitochondria, dilated cisternae of ER, and the nuclear membrane (depending on time of incubation, fixative, concentration of sinigrin). The results varied using different fixatives, time of incubation and concentration of the substrate, but under optimal conditions, the enzyme activity was mainly found in the cisternae. The enzyme was not confined to any specific type of cells, being detected in the majority of cells of the root tip (root cap, zone of elongation and root hair zone). The precipitation in the dilated cisternae of the ER suggested that the enzyme was **membrane-associated.**

Pihakaski and Iversen (1976), using a cell fractionation technique on *Sinapis alba* roots, localized the myrosinase activity to the dictyosomes and the smooth ER, which again supported the theory of the membrane-association of myrosinase. Further, Maheshwari *et al.* (1981), using different fixation mixtures, and the fact that lead sulphate deposits were formed at the site of myrosinase activity, showed that myrosinase was detected in the majority of the cells of the embryo (cotyledons), and appeared to be localized intracellularly in the **plasma membrane.**

Evidence for the sub-cellular, membrane-bound, localization of myrosinase was demonstrated by Lüthy & Matile (1984), who presented a model for the sub-cellular compartmentation of myrosinase-glucosinolates. Using intact vacuoles (by subcellular fractionation), and hexose-6-phosphatase as a cytosolic marker, suggested that myrosinase was present in the cytoplasm with a remarkably tendency to adhere to membrane surfaces (with a variation of affinity to different membranes), while glucosinolates were found only inside the vacuoles. Thus, myrosinase and glucosinolates are localized in the same cell, but are separated by the tonoplast; the enzyme myrosinase cannot react with the vacuolar glucosinolates unless the vacuoles are disrupted-the so called 'mustard oil bomb' (see figure-1.11-).

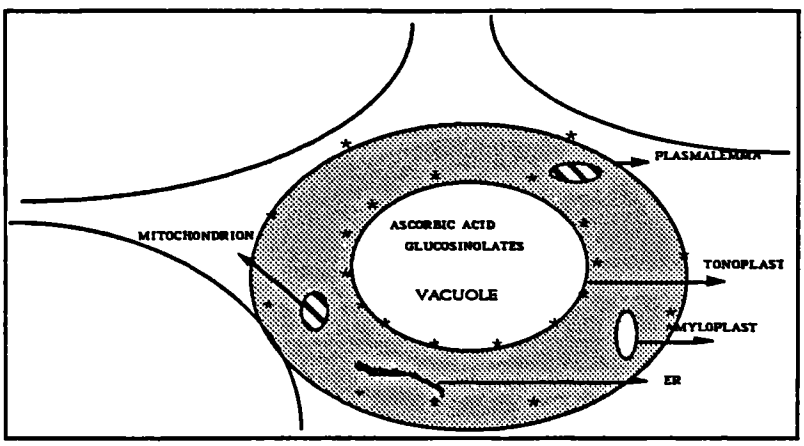


Figure -1.11- The mustrard oil bomb. The model for subcellular compartmentation of glucosinolates, L-ascorbic acid and myrosinase (after Lüthy and Matile, 1984).

* myrosinase location

1.9.4.2. Localization in specific cells, myrosin cells

Heinricher (1884), although not using the term myrosin cells, first discovered the existence of cells which stained intensively with Millon's reagent, thus establishing their proteinaceous nature. Guignard, (1890), first coined the name **myrosin cell,** reflecting the view that these idioplasts contain myrosinase; he found that the tissue containing such idioplasts liberated mustard oil when added to a solution of the glucosinolate sinigrin, thereby indicating that these cells contained the enzyme myrosinase. Spatzier (1893) first coined the name **myrosin grains,** concluding that these grains were different from the grains found in the surrounding cells.

Rest and Vaughan (1972), showed that cotyledons of the embryo of the mature *Sinapis alba* seed contained two types of cells (aleurone, myrosin cells) and that myrosin cells had certain globular structures (**myrosin grains**) which stained with Millon's reagent more intensively, indicating the higher proportion of tyrosine, tryptophan and phenolic compounds in them. They followed their production and showed that they had been produced by a coalescing of small vacuoles which produced one vacuole filled with protein. There was a similar content (stain) in the myrosin grain and the ER cisternae, indicative of the their similarity. Werker & Vaughan (1974), using saphranine fast green and Millon's reagent, detected myrosin cells in cotyledons and hypocotyls 4-72h after imbibition of *Sinapis alba* seeds. After 36h in water, myrosin grains appeared as separate-vacuole filled with myrosin. Later, the tonoplast between the variously-sized vacuoles became gradually no longer visible, and one big central vacuole filled with myrosin was formed. At 72h, it was sometimes difficult to discern a tonoplast separating the myrosin from the cytoplasm. The individual myrosin grains of the embryo each developed into a separate vacuole, which in turn coalesced to form one central vacuole filled with myrosin (myrosin accumulates in the ER cisternae before being transferred to the vacuole). No new myrosin cells were developed during germination; they were already produced in the embryo in the developing seed. Further, Werker & Vaughan (1976), again using *Sinapis alba,* detected myrosin cells in mesophyll cells of the leaves and parenchyma cells of the stem adjacent to the vascular cylinder, using Millon's reagent, saphranine and lactophenol aniline blue. In the same study, they followed the development of myrosin grains in cells of the leaf blades and demonstrated that swollen ER cisternae form vesicles, which later form the vacuoles which coalesce to produce myrosin grains.

Iversen *et al.*. (1979), working with *Sinapis alba,* and using the phase contrast microscope, showed that myrosin cells were present in the radicle after 2h, in the taproot after 24h, and in the root tip after 48h of imbibition respectively. After 24h, a hydrolysing process was initiated and only myrosin-like cells could be detected. The main organelles in myrosin cells were the spherical myrosin grains which were assumed to be separate vacuoles filled with protein. They were found in the cortex, being located in 4% of the cortical cells. However, when myrosinase activity was compared with the existence of myrosin cells, there was no correlation. Phelan & Vaughan (1980) supported the same conclusion; they found myrosinase activity in organs that lacked myrosin cells (root or flower parts of *Sinapis alba*), although there was a good correlation between myrosin cells and myrosinase activity in developing seeds and very young seedlings.

Iversen & Baggerud (1980) also showed that differentiated Brassica tissue was not a prerequisite for the detection of myrosinase activity; they observed that protoplasts showed increased myrosinase activity compared with that of intact tissue.

Phelan *et al.* (1984), took advantage of the fact that an electron-dense deposit of barium sulphate forms at the site of hydrolysis of glucosinolates, and detected myrosin cells in all parts of the axis of the young root of *Raphanus sativus* after 1h, 24h and 48h of imbibition, but after 72h, when the separate regions could clearly be seen, they could no longer be distinguished in the root tip or root hair zone, although they were still abundant in the hypocotyl.

Bones & Iversen (1985) in a detailed study on the existence and distribution of myrosin cells in hypocotyls, cotyledons and roots of very young plants of seven species of the Brassicaceae, detected myrosin cells in the hypocotyls up to 48h imbibition, in cotyledons up to 192h and in roots up to 42h, using different stains(toluidine blue, methylene blue azur II, lactophenol aniline blue and fuchsin). They did not observe *de novo* synthesis, or division of myrosin cells, and distribution of myrosin cells decreased with age. Myrosin grains were not the prerequisite for myrosinase activity, because callus cultures from stem segments exhibited myrosinase activity, but myrosin cells were not detected in these same cultures.

In 1990, Thangstad *et al.* showed, using an antibody to myrosinase, a specific positive localization of myrosinase in the radicle of rapeseed imbibed for 4h. With few exceptions, the positive-reacting cells in the radicle were located in the second outermost layer of the root cortex. The myrosinase was associated with the single membrane surrounding the myrosin grains. After 48h of imbibition, less myrosin cells were detected. This is in agreement with their decreasing number during germination.

Höglund *et al.* (1991), using a monoclonal antibody to myrosinase, showed that the enzyme was present in a small number of cells of the developing seed of *Brassica napus.* The distribution of myrosinase as seen by immunocytochemistry was compared to that observed when the traditional technique for the detecting myrosin cells, viz. Millon's reagent, was used on consecutive sections of rapeseed embryo taken 39 days after pollination. Evidently, the two procedures identified the same set of cells; in the roots, the myrosin cells were mainly located in the cortex, in the stem: in xylem and cortex, and in young foliage leaves in: guard cells, parenchyma and vascular tissue close to phloem. The antibody localized the enzyme in the cytoplasm, whereas Millon's reagent stained the interior of the myrosin grains. Millon's reagent reacts with tyrosine and tryptophan residues i.e. compounds which contain an aromatic ring structure. Some of the glucosinolates, viz. the methylidolyl's, have structures similar to tryptophan and hence, are probably the reactants detected by Millon's reagent.

Bones *et al.* (1991), using immunocytochemical techniques and *Brassica napus* , showed specific labelling of protein bodies in myrosin cells, and localized the enzyme in all parts of the radicle after 4h imbibition. A large proportion of the labelled cells were found in the marginal zone, both in radicles and hypocotyls. The morphology of the myrosin cells changed dramatically during seedling growth. The large fluorescent grains that were detected at 4h after imbibition, were fused to form small spots. Earlier studies of myrosin cells and their development had relied on general staining techniques (i.e. Millon's reagent, saphranine, lactophenol blue). Due to a dilution of the content of the myrosin grains, using a non-specific stain, it was only possible to follow the development of the myrosin cells during early seedling growth (i.e. only up to 72h after imbibition). Using specific antibodies, the development of myrosin cells appears

to follow a similar pattern in all parts of the plant i.e. cotyledons, hypocotyls and root. This development seemed to include fission, followed by formation of small myrosinase-containing grains. The myrosinase was not uniformly distributed in the vacuoles, but in small vesicles. At later stages it appeared only in the periphery of the vacuole.

Thus, using a immunofluorescent technique, Thangstad *et al.* (1990) and Höglund *et al.* (1991) showed that myrosinase was membrane-associated.

In 1991 (Thangstad *et al.*), and in 1992 (Höglund *et al.*), two reports localized the enzyme myrosinase at the subcellular level in imbibed seeds of 4 species of Brassicaceae and embryos of *Sinapis alba* respectively, using the powerful technique of immunogold labelling. Thangstad *et al.* (ibid.), localized the enzyme in specific cells in myrosin grains in radicles and cotyledons of 24h imbibed seeds, and Höglund *et al.* (ibid.), also localized the enzyme in the interior of myrosin grains in myrosin cells. Myrosin grains first appeared at 27 days after pollination. Thus localization at the subcellular levels (immunogold labelling) revealed that myrosinase was inside the grains.

2. Materials and methods

2.1. Media

All glassware and media were autoclaved at 103 KPa/120°C for 20 min. Glass distilled water was used to prepare the media .

Potato Dextrose Agar

Potatoes (fresh weight)	200 g
Dextrose	20 g
Agar	20 g
S.d.w.	1 l

(Johnston & Booth, 1983)

Slices of pre-washed, organically grown potatoes (Safeway): 200 g were boiled in 500 ml of distilled water for 45 min and the contents filtered through muslin cloth. Twenty g of agar (Oxoid) was boiled until dissolved in 500 ml of distilled water and mixed with the potato broth after adding dextrose (Glucose, Sigma). The mixture was made up to 1 l. Potato broth was prepared as above without adding agar.

Water Agar Medium

Water agar medium was prepared by adding 20 g of agar (Oxoid) in 1 l of distilled water.

Re-isolation Medium

Re-isolation medium was prepared by cooling down PDA medium after autoclaving and adding 0.25 g streptomycin (Sigma) diluted in 5 ml methanol to prevent bacterial growth.

Hall and Ly's Medium

This medium was prepared by adding:

Magnesium sulphate $MgSO_4$ $7H_2O$	0.5 g (BDH),
Zinc sulphate $ZnSO_4$ $7H_2O$	0.5 mg (BDH),
Manganese sulphate $MnSO_4$ H_2O	0.5 mg (BDH),
Copper sulphate $CuSO_4$ $5H_2O$	0.16 mg (BDH),
Potassium dihydrogen phosphate KH_2PO_4	1 g (BDH),

Dipotassium hydrogen orthophosphate K_2HPO_4	0.9 g (BDH),
Potassium Nitrate KNO_3	2 g (BDH),
$NaMoO_4$ H_2O	0.01 mg (BDH),
Glucose	0.6 g (Sigma)
Agar	20 g (Oxoid)
S.d.w.	1 l

(Hall and Ly, 1972)

The solution of the phosphate salts (13 mM) was prepared and autoclaved separately from the other components of the medium, then added before pouring. This medium, diluted by a factor of 30, was used by Horiuchi *et. al.* (1990), to establish characters which reliably distinguished the groups pathogenic to Solanaceous plants (*V.dahliae* isolates obtained from different hosts: groups A, B, C) from those which were non-pathogenic to Solanaceous plants but pathogenic to Crucifers (group D) based upon the morphology of microsclerotia produced.

Oilseed rape Agar Medium.

Oilseed rape	50 g
Agar	20 g
S.d.w	1 l

(Peterka & Schlosser, 1989)

Oilseed rape agar medium was prepared by blending 50 g oilseed rape fresh material (leaves and stems of one-month-old plants) in a waring blender for 10 min and the content filtered through four layers of muslin cloth. After adding 20 g of agar (Oxoid) the volume was made up to 1l.

Prune Extract Medium.

Prune extract	100 ml
Lactose	5 g
Yeast extract	1 g
Agar	20 g
S.d.w.	1 l

(Talboys, 1960).

The pH of the medium was adjusted to 5.8-6.0.

Concentrated prune extract was prepared by simmering 50 g chopped dried prunes with 500 ml of water until they were soft. The suspension was then filtered through muslin, made up to 1 l, sterilised and stored at 4°C until required. This medium was proposed by Talboys (1960) to enable a ready microscopic identification and distinction between *Verticillium albo-atrum* and *Verticillium dahliae* .

Eckert's Medium (modified)

Eckert's medium (modified by Howell, 1970) was prepared by adding:

Bactopeptone	5 g (Oxoid),
Bacto yeast extract	3 g (Oxoid),
Potassium dihydrogen phosphate KH_2PO_4:	1.36 g (BDH),
Di potassium hydrogen orthophosphate K_2HPO_4	1.68 g (BDH),
Magnesium sulphate $MgSO_4$ $7H_2O$	0.5 g (BDH),
Agar	20 g (Oxoid) ,
Glucose	20 g (BDH)
Sucrose	5 g (Sigma)
S.d.w.	1 l

(Eckert, 1962; Howell, 1970)

Medium for Extracellular Polyphenol Oxidase (p.p.o.) Activity

Eckert's medium modified by Howell (ibid.), was cooled down after autoclaving and amended with filter-sterilised tannic acid (Sigma) to achieve a final concentration of 0.1% w/v. This was achieved by adding 1 ml of 4 % w/v aqueous, filter-sterilised tannic acid to each Petri dish containing 40 ml of Eckert's modified medium. It was very important to incorporate tannic acid after autoclaving the medium because tannic acid lowered the pH to 5.2 and did not allow the agar to solidify satisfactorily.

This medium was used by Howell (1970), to differentiate haploid isolates of *V.dahliae* from cotton and diploid forms derived from the corresponding haploids, according to their *in vitro* polyphenol oxidase activity which he showed was related to their pathogenicity to cotton. Also, Horiuchi *et. al.* (1990), used this medium to differentiate group D (*V.dahliae* isolates obtained from Cruciferous plants and non-pathogenic to Solanaceous plants) and the other groups A, B, C (obtained from different hosts and pathogenic to Solanaceous hosts) based upon the same enzyme test but with reverse results.

Medium for polygalacturonase activity

Modified Eckert's medium was incorporating with 0.5 % v/v galacturonic acid (BDH), pH 5.0, in place of sucrose. This medium was used by Howell (1970) to differentiate haploid and diploid forms derived from the haploids forms of *V.dahliae* from cotton by their *in vitro* polygalacturonase activity which he showed was related to their pathogenicity to this host.

Czapek-Dox Medium.

Czapek-Dox liquid medium was prepared by adding:

Sodium nitrate $NaNO_3$	2 g (BDH),
Potassium dihydrogen phosphate KH_2PO_4	1 g (BDH),

Magnesium sulphate $MgSO_4$ $7H_2O$	0.5 g (BDH),
Potassium chloride KCl	0.5 g BDH),
Ferrous sulphate $FeSO_4$ $7H_2O$	0.01 g (BDH),
Zinc sulphate $ZnSO_4$ 7 H_20	0.01 g (BDH),
Copper sulphate $CuSO_4$ $5H_20$	0.005 g (BDH),
Sucrose	30 g (Sigma),
S.d.w.	1 l

(Johnston & Booth, 1983).

Complete Medium

Complete medium was prepared by amending the minimal medium (Czapek-Dox) with:

Casein hydrolysate	2 g (Oxoid),
Mycological peptone	2 g (Oxoid),
Malt extract	2 g (Oxoid),
Yeast extract	2 g (Oxoid),
Agar	20 g,
S.d.w.	1 l

Haploidization medium

Haploidization medium was prepared by adding 0.02 M chloral hydrate (BDH) in complete medium.

2.2. General techniques

2.2.1. Culture of the pathogen

Isolates of *Verticillium sp.*, previously obtained from different hosts and geographical locations, were maintained as single-spored isolates on PDA slopes in the dark at 4 °C in three different locations. All isolates were transferred to fresh PDA slopes every 8 months. For experiments, 1 mm^3 of fungal inoculum was transferred on to each PDA Petri -dish and grown in the dark at 24-25 °C until required. Descriptions of the isolates of *Verticillium sp.* used are presented in table -2.1b-. The isolates of *V.dahliae* used were maintained under conditions of UK MAFF licence No PHF 148B/523/43. All infected host material, used vermiculite and cultures of the pathogen, were destroyed by autoclaving at completion of each experimental test.

2.2.2. Preparation of single-spore isolates of the pathogen

Verticillium cultures were grown for 7 days on PDA at 24-25 °C in the dark. Spore suspensions were prepared by washing off the 7 days-old parent plate and diluted and then adjusted to give $1X10^6$ spores / ml using an haemocytometer (Neubauer Brightline, improved double, BDH). The suspension was further diluted to give approximately 25 spores /ml and 2 ml in s.d.w. was used to seed the entire surface of a Petri dish containing PA medium (1% agar). After one day's incubation at 24-25 °C in the dark, conidia were seen to have germinated (observed under a dissecting microscope) and were picked up as single-spored colonies using a sterile inoculating needle. These single-spored colonies were further grown on fresh PDA plates. Five independent 'daughter' isolates were kept from each 'parental' population, to verify the results obtained for each isolate.

2.2.3. Semi-synchronous, spread-plate cultures

Spore suspensions were obtained by washing off the surface of 7 day-old parent PDA culture using 2 ml of sterile distilled water and this was used to seed the entire surface of a PDA plate. These spread-plate cultures were incubated for 7 days at 25 °C in order to produce a relatively uniform population of conidia with respect to size and age.

2.2.4. Preparation of spore suspensions

Spore suspensions were prepared in s.d.w. from 7 day-old, densely-conidiating, PDA spread cultures. Spores were harvested by the addition of 5 ml s.d.w. and gently agitated by slow hand movement of the plate to release spores. The resulting spore suspension was diluted if necessary (concentrations above 10^6 spores/ml gave a white-milky colour to the suspension) and adjusted to $1x10^6$ spores/ml using an haemocytometer (Neubauer Brightline, improved double, BDH) with configurations: 0.1 depth and 1/400 mm^2 area of the smallest square. One chamber of the haemocytometer was carefully flooded with 200 µl of spore suspension and the coverslip was moistened by exhalation and pressed firmly over the counting chambers until colour interference patterns were visible. Counts were made using a Leitz microscope (Dialux 20, E.Leitz Instruments Ltd., Luton, UK) with a X 50 objective. Two replicate counts were made by using the formula:

(Number of spores counted X 4 X 10^6) / (Number of squares counted X 16)= spores/ml

and the average value was calculated.

2.2.5. Spore length measurements

Spore suspensions were prepared in s.d.w. from 7-day-old semi-synchronous spread PDA cultures. One drop of 1 X 10^6 spores / ml was pipetted onto a glass slide, a coverslip was placed on top, and spores were observed using a Leitz microscope (Dialux 20, E.Leitz Instruments Ltd., Luton, UK.) with a X100 water-immersion objective. Measurements were based on average of 50 readings for each isolate using a

calibrating eye-piece graticule (Graticules Ltd., Tunbridge, UK). The eye-piece graticule showed 100 units. These units were calibrated to μm by means of a stage micrometer (100 X 0.01 mm =1 mm). One unit on the eye-piece (EPU) corresponded to 1.1μm on the stage micrometer (under these magnification conditions) which remained constant in future observations.

2.2.6. Tests of spore viability using fluorescein diacetate (FDA)

FDA is an excellent indicator of cell viability tested in plants, fungi, and mycobacteria, Butt *et al.* (1989). The principle of staining with FDA relies on the non-polar FDA molecule crossing the plasma membrane and its ester bonds being hydrolysed in the living cytoplasm by a non-specific esterase to release the polar fluorescent fluorescein as a free molecule which accumulates in the cytoplasm because it can not pass through either the plasma membrane or the tonoplast of living cells, (Helsop -Harrison & Helsop-Harrison, 1970). Living cells therefore are distinguished by their bright, stable, yellowish-green fluorescence when irradiated with UV light. Vacuoles of healthy cells do not take up fluorescein and therefor appear dark. Non-viable spores (lacking detectable levels of esterase and possessing damaged plasma membrane allowing leakage of cell components) show no , or very slight indications of fluorescence.

FDA was diluted in acetone at the concentration of 5 mg/ml and used at the final concentration of 0.01% w/v. S.d.w. was used to dilute the stock solution that stored at -20 °C until required.

A spore suspension was prepared by washing from a 14-day-old spread culture. The spores were harvested and adjusted to the final concentration of $1x10^6$ spores/ml. The sample was used immediately or kept at -4°C for 24h to test the viability of spores when half of the spore suspension was autoclaved at 103 kPa at 120 °C for 20 min to be tested as a control, non-viable comparison. An aliquot of 2μl FDA and 200 μl of spore suspension were mixed on a slide, a coverslip was placed on top and left for 6 min. The spores were examined under ultra violet light with a Leitz Dialux 20EB epifluorescence microscope equipped with a Phloemopak 25 fluorescence vertical illuminator supplying UV light from a 50 W high pressure mercury lamp. A fluotar water immersion lens[1] was used to observe the treated spores and I2 filter block[2].

Table -2.1.a- Hosts of isolated pathogens

Non-Crucifer		Crucifer	
Tomato	*Lycopersicon esculentum*	Oilseed rape	*Brassica napus*
Potato	*Solanum tuberosum*	Chinese cabbage	*B.cam.var.pecinensia*
Eggplant	*Solanum melongena*	Brussels sprout	*B. ol.var.gemifera*
Olive tree	*Olea europaea*	Wild Radish	*Raphanus sativus*
Clover	*Trifolium pratense*	Shepherd's purse	*Capsella.b.pastoris*
Lucerne	*Medicago sativa*	Stock	*Matricaria maritima*
Cotton	*Gossipium barbadence*	Horse radish	*Cochlearia armoracia*
Sugar beet	*Beta vulgaris*		

[1] NPL FLUOTAR Type of magnification: 100 / numerical immersion apperture: 1.20 W
Tube length: 160/ Coverslip thickness: 0.17

[2] Exciting filter: BP 450-490 nm, suppression filter: LP 515 nm

Table -2.1.b-Isolates of *Verticillium sp.* used in this investigation

HOST	PATHOGEN *	COUNTRY	YEAR	ISOLATE
Sugar beet	*V. d.longisporum* [1]	Sweden	1981	161
Oilseed rape	*V.d.longisporum* [1]	Sweden	1981	162
Wild radish	*V.d.longisporum* [2] ?	Japan	1980	86207
Chinese cabbage	*V.d.longisporum* [2] ?	Japan	1980	84013
Chinese cabbage	*V.d.longisporum* [2] ?	Japan	1980	84120
Brussels sprouts	*V.dahliae* [3]	England	1964	111
Horse radish	*V.d.longisporum* [4]	Germany	1961	195
Clover	*V.dahliae*	Germany	1987	G1
Oilseed rape	*V.dahliae*	Germany	1987	G10
Potato	*V.dahliae*	Germany	1988	G16
Stock	*V.dahliae*	Germany	1989	G17
Oilseed rape	*V.dahliae*	Germany	1989	G19
Oilseed rape	*V.dahliae*	Germany	1989	G25
Oilseed rape	*V.dahliae*	Germany	1990	G22
Oilseed rape	*V.dahliae*	Germany	1990	G29
Oilseed rape	*V.dahliae*	Germany	1990	G34
Oilseed rape	*V.dahliae*	Germany	1991	GV165
Oilseed rape	*V.dahliae*	Germany	1991	GV166
Oilseed rape	*V.dahliae*	Germany	1991	GV167
Oilseed rape	*V.dahliae*	Germany	1991	GV168
Oilseed rape	*V.dahliae*	Germany	1991	GV169
Oilseed rape	*V.dahliae*	Germany	1991	GV170
Oilseed rape	*V.dahliae*	Germany	1985	G334
Oilseed rape	*V.dahliae*	Germany	1988	G859
Oilseed rape	*V.dahliae*	France	1991	F1
Oilseed rape	*V.dahliae*	France	1988	F617
Oilseed rape	*V.dahliae*	France	1988	F617-7
Oilseed rape	*V.dahliae*	France	1988	F617-9
Oilseed rape	*V.dahliae*	Poland	1989	P671
Oilseed rape	*V.dahliae*	France	1989	F654-2
Oilseed rape	*V.dahliae*	Germany	1988	G856=S856
Olive tree	*V.dahliae* [5]?	Greece	1992	GR1
Eggplant	*V.dahliae*	Italy	1976	140
Tomato	*V.dahliae*	USA	1976	130
Tomato	*V.dahliae*	USA	1976	133
Cotton	*V.dahliae*	Spain	1988	C13
Shepherd's purse	*V.dahliae*	USA	1976	362
Chrysanthemum	*V.albo-atrum*	UK	1964	230
Lucerne	*V.albo atrum*	France	1981	234

*Identification as supplied.

[1] The ploidy of 161,162 had been verified and published by Jackson & Heale, 1985, therefore referred as *V.d.longisporum* Stark (Stark,1961).
[2] Described by Horiuchi *et.al.* 1990, as having similarities with *V.d.longisporum.*
[3] Described by Isaac, 1957, as infecting Brussels sprouts in the Eversham area.
[4] This was first isolated and named *V.d.longisporum* by Stark (1961) and its diploidy later investigated by Ingram, (1968); Typas & Heale, (1977, 1980); Hastie & Heale, (1984); Jackson & Heale, (1985); Horiuchi *et.al.*, (1990) and Baig, (1991).
[5] Supplied as a peculiar isolate of *V.dahliae* by Prof. E..C.Tjamos(1992), Plant pathology lab, Agricultural University of Athens.

2.3. Pathogenicity tests

2.3.1. Disinfection and germination of seeds

Oilseed rape seeds were surface-sterilised/disinfected using a modified method of Sauer and Burrough (1986) by rinsing with 50% methanol and then with 5% commercially obtained bleach (Domestos) for 3 min to prevent the growth of saprobic fungi. Finally, they were rinsed 3 times with s.d.w. and germinated on moist filter paper (Whatman No 3) in Petri dishes for three days in the dark at 24-25 °C.

2.3.2. Cultivation of oilseed rape and Chinese cabbage

Germinated seedlings of cultivars of oilseed rape (table -2.2-) and Chinese cabbage (F1-TipTop 1902) were grown further in seedling plastic pots (1.5 inch) containing vermiculite in semi-controlled environment greenhouse conditions, supplemented with Atlas 400W Kolarc MBFI mercury vapour lamps, lighting design, producing 27,000 lumens at a photosynthetic photon lux density of 120 $\mu Em^{-1} s^{-2}$ and a light regime of 8 hours darkness, (minimum night temperature of 15°C) and 16 hours light, with day temperatures in the range of 15-25°C .

Treated or untreated, 2-week-old seedlings of oilseed rape were transferred to 1.5 inch plastic pots containing vermiculite. The Chinese cabbage seedlings were transferred to 2.5 inch plastic pots containing a potting mixture (John Innes Compost No 1, Levingtons Potting mixture Compost and washed coarse sand in a ratio of 1:1:1).

The oilseed rape seedlings were fed twice per week with 1/2 strength of Phostrogen liquid feed. All plants were watered at least 3 times a week.

Five, winter oilseed rape cultivars of double low (low erucic acid and low glucosinolate level) type sown Table-2.2-, were used to test the pathogenicity of isolates of *V. dahliae* obtained from Crucifer and non-Crucifer hosts. Cultivar Cobra had become outclassed but the other four were fully recommended for general use (NIAB, 1993).

2.3.4. Inoculation

2.3.4.1. Seed inoculation

Surface-sterilised seeds of cultivar Cobra were immersed in spore suspensions of the respective pathogen isolate at a concentration: $1x10^6$ spores/ ml for 1 min with occasional shaking.

Forty seeds and one tray was employed for each treatment, each tray being sown with 20 seeds. Seeds were inoculated with either a virulent isolate of the pathogenic strain of *V.d.longisporum* , (161, sugarbeet), or an avirulent isolate of the non pathogenic strain *V.dahliae*, (130, tomato). For controls the surface-sterilised seeds were immersed in sterile distilled water.

2.3.4.2. Root inoculation.

For each experimental run one hundred and twenty, 2-week-old oilseed rape seedlings were carefully uprooted, washed with s.d.w. and the root systems immersed in either a spore suspension of the appropriate isolate containing $1x10^6$ spores/ ml for 15 min with occasional shaking or left uninfected (controls). One inoculated seedling was transferred into each 1.5 inch pot and 24 (3 X 8) replicate pots employed for each isolate under test. For control tests (uninoculated), the seedlings were dipped in s.d.w. and similarly planted. The pots were placed randomly in the green house to ensure that the location was not a factor in the development of the plant or the disease. The pattern followed was: Each of 3 seedling trays was consisted of 40 pots in which were transferred the inoculated and control seedlings under test. Of these 8 replicate seedlings were employed for each treatment (5 different treatments i.e. four isolates under test and one uninoculated control), i.e. 5 X 8 pots were employed. This test was performed in three replicate trays. This method allowed the present writer to test all isolates in table-2.1b- against oilseed rape cultivar Cobra and also the other four oilseed rape cultivars listed in table-2.2- against four other isolates of the fungus.

Table -2.2-. Cultivars of oilseed rape under investigation.

	COBRA	ENVOL	IDOL	SAMOURAI	FALCON
BREEDER ORIGIN	Germany	France	France	France	Germany
GLUCOSINOLATE *	17.6	14.9	17.1	15.0	15.2
OIL % (SEED)	42.0	44.3	44.0	43.5	42.8
YIELD	low	high	average	average	low
MATURITY	early	early	early	very early	early
STEM	weak	weak	weak	strong	weak
STEM CANKER[1] **	susceptible (4)	less susceptible (5)	less susceptible (5)	susceptible (4)	less susceptible (5)
LIGHT LEAF SPOT[2] **	susceptible (4)	Less susceptible (6)	resistant (7)	susceptible (5)	less susceptible (6)
DOWNY MILDEW[3] **	resistant (7)	resistant (8)	resistant (8)	resistant (7)	resistant (7)

*µm/g of seed, **1-9 scale disease resistance.

[1] Canker (*Leptosphaeria maculans*, asexual stage *Phoma lingam*), causes leaf spotting over winter and cankers on the stem later.

[2] Light leaf spot (*Pyrenoperiza brassicae*, asexual stage *Cylindrosporium concentricum*),causes light green or bleached lesions on the leaves.

[3] Downy mildew (*Peronospora parasitica*) causes yellow discoloration of the upper leaf surface and white fungal growth on the lower surface.

Table-2.3-. Example: Lay-out of seedling pathogenicity test

SEEDLING PATHOGENICITY TRAY

A	D	B	D	B
C	B	C	A	D
A	C	A	B	C
C	B	D	D	A
B	D	C	E	E
E	E	A	C	E
B	C	E	D	D
E	A	B	A	C

Treatments: **Five**

Replicates: **Eight (seedlings)**

Repeats: **Three (trays)**

3 X (8 X 5)=120

A	*V.dahliae*	isolate 130
B	*V.d.longisporum*	isolate 161
C	*V.dahliae*	isolate G859
D	*V.dahliae*	isolate G334
E	Control	S.d.w.

2.3.5. Re-isolation of the pathogen

2.3.5.1. Moist chamber

Pieces (4-5 mm) of the stems and the roots of inoculated and control plants, twenty days after inoculation, were surface-sterilised by immersing them for 2 min in a solution of 5% Domestos. The plant material was then rinsed in s.d.w. and approximately 1 mm at the ends of each piece was removed with a sterile scalpel. The pieces were transferred to Petri-dishes containing sterile moist paper and incubated in the dark at 24-25 ^{0}C for 5-10 days. For verification, a dilution series was prepared from conidial heads and plated on PDA medium. The formation of typical verticilliate conidiophores and later, black microsclerotia, gave confirmation of the presence or absence of *V. dahliae.*

2.3.5.2. On water agar medium

Sterile pieces of stem and root material (as above) were also transferred to water agar plates and incubated in the dark at 25°C for 5-10 days. Microscopic examination again verified the presence or absence of *V. dahliae*. Conidial lengths and type of microsclerotia gave indications of *V.d.longisporum* or *V.dahliae.*

2.3.5.3. On PDA medium

To avoid bacterial contamination, re-isolations performed for the detection of the extent of colonization of the isolates tested were carried out on re-isolation medium.

2.3.6. Seedling pathogenicity tests under semi-controlled greenhouse conditions

The pathogenicity of 29 isolates of *V.dahliae* and *V.d.longisporum*, originally obtained from different hosts and countries (tables-3.20-, 3.21-, 3.22, see results section) was tested against one oilseed rape cultivar: Cobra. To verify the pathogenicity of Cruciferous isolates of *V.dahliae*, 4 different isolates were also tested against four different oilseed rape cultivars (table-2.2-). Sterilised seeds of Cobra were germinated for 72h on sterilised filter paper moistened with s.d.w. and those with emerged radicles were transferred into vermiculite for 11 more days before inoculation. Seedlings were grown under partially environmentally-controlled greenhouse conditions.

Spores were washed from a spread, 2-week-old culture grown in PDA in thé dark at 24-25°C using 10 ml of s.d.w. Prior to inoculation, seedling roots were rinsed carefully in s.d.w. Transplanting injury to root endings inevitably exposed the vascular system and increased infection. Twenty-four control seedlings were root-dipped in s.d.w. Test seedlings were transferred to fresh vermiculite and placed in the greenhouse with the environmental parameters and lay-out described previously (Section 2.3.4.2; Table-2.3-, page s87, 88).

Symptoms were assessed at 11 days, and subsequently at 15, 18, 20, 23, 25, 27 and days after inoculation, using the following key :

0. No symptoms.

1. First true leaves with small petioles as compared with controls.

2. One or two cotyledons yellow (chlorotic).

3. Two cotyledons brown (necrotic); stunting of plant, leaves start becoming yellow (chlorotic).

4. 50 % of the adult leaves show severe symptoms (chlorosis/necrosis).

5. All plant parts necrotic and dead.

Mean disease scores were calculated for each isolate/cultivar combination

Empty plastic seed trays covered with heavy metal wire to support seedling pots enabled inoculation of several isolates without cross contamination during watering operations.

2.3.7. Extent of colonization by pathogenic isolates

Seedlings of inoculated oilseed rape (cultivar Cobra) were sampled at 6, 9, 13, 15 and 20 day intervals after inoculation.

Disease scores were recorded at the time when the plants were removed from the potting mixture. The stem of each plant was cut above the soil surface and below the cotyledons. The sections were surface-sterilised by immersion in 5% Domestos and the ends of each piece were removed. Each plant stem sampled was subdivided into 5 pieces and transferred into a Petri-dish with re-isolation medium in a specific order according to the distance from the root. The plates were kept at least 2 weeks at 25 ^{o}C in the dark and examined for the presence of the pathogen under a dissecting microscope.

Two series, each of 10 replicate plants for each day sampled, inoculated with each of the two *Verticillium* isolates under test (130, 161) respectively, were employed.

Recovery of *V. dahliae* was only considered as positive when microsclerotia production was detected together with verticilliate conidiophores. For identification of *V.d.longisporum* conidial lengths were measured.

2.3.8. Microscopic examination of penetration and development of the pathogen in the root of oilseed rape cultivar Cobra.

Two-week-old oilseed rape (cultivar Cobra) seedlings were inoculated by the root-dipping technique with either of two isolates viz.130, *V.dahliae* from tomato USA (avirulent) and 161, *V.dahliae* from sugar beet, Sweden (virulent), under greenhouse conditions .

The seedlings were root-dipped for 15 min in the appropriate spore suspension under standard conditions and then transferred into vermiculite for 1 ,3,10, and 15 days. Then the roots were rinsed thoroughly with s.d.w. to remove grains of vermiculite and prepared using the following procedure. Control roots were dipped in s.d.w.

The roots were cut into pieces 3-5 mm long and fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH : 7.1) at room temperature overnight and washed in three changes of the same buffer for 30 min at 4°C. After the third wash, they were post-fixed in 1% osmium tetroxide at 4 ^{o}C for 2h and dehydrated in ethanol at increasing concentrations (30% for 20 min, 50% for 20 min, 70% for 30 min, and three times in 100% for 30 min). They were then washed twice for 10 min in propylene oxide. After the second wash in propylene oxide, they were embedded in resin by gradual infiltration (1h in 25/75 resin/propylene oxide, 1h in 50/50 resin/propylene oxide, 4h in 75/25 resin/propylene oxide and finally in 100% resin overnight). Following this, they were put in fresh 100% resin and hardened in an oven at 60 °C for 48h and cut with glass knives on a Reichart FC2 microtome (0.5-1.5μm). Samples were stained in 1% Toluidine blue (a metachromatic agent) in 0.5% Borax and studied under the light microscope (Leitz Dialux 20EB microscope). This stain is not a tissue-specific stain; it produces various shades of blue/green allowing the identification of tissue components as well as fungal structures by their different degrees of colour contrast.

2.3.9. Field observations of *V.dahliae* in oilseed rape in Germany (Rostock, Malchow/Poel, Hohenlieth, Ferman).

A visit to Germany was performed in order to make field observations of Verticillium disease in oilseed rape. Observations were made in 3 different fields: 1) In Rostock were the plants were naturally infected with the pathogen, 2) in Malchow in which the soil was artificially infested with microsclerotia of the fungus and 3) in Hohenlieth where the soil was also artificially infested with microsclerotia, plus infected residues of oilseed rape.

2.4. Characterization of the pathogen

To identify and characterize the different isolates of *Verticillium sp.* and in particular to separate haploid *V.dahliae* Kleb. strains from the diploid *V.d.longisporum* Stark strain, a combined set of morphological, enzymatic and genetical criteria were used. Morphological characters which were used as criteria for identification were the typical conidiophores, conidia and microsclerotia examined using both the light and dissecting stereo-microscope. Enzymatically, isolates were characterized by their ability to exhibit extracellular polyphenol oxidase and polygalacturonase activity on selected media. Genetic characters were studied by the use of molecular biology methods and by measuring the DNA content of ungerminated conidia of each isolate by the use of Feulgen microdensitometry. Two different molecular biology methods were used: The polymerase chain reaction (PCR) for *in vitro* randomly amplified polymorphic DNA (RAPDs) and Pulsed Field Gel Electrophoresis (PFGE) for Chromosome Size Length DNA Polymorphisms.

2.4.1. Morphological differences between isolates

2.4.1.1. The morphology of microsclerotia on different media

The formation of resting structures (microsclerotia) was studied on four different media in Petri-dishes (9 cm diameter, containing 40 ml of medium).

Inocula (1 mm cube) from the margin of a 14-days-old culture on Hall and Ly's medium were placed on Hall's and Ly's medium diluted by a factor of 30 or undiluted, with 2% agar and incubated for 20 days at 25 ^{o}C in the dark, to study the formation of microsclerotia. The same procedure was repeated using oilseed rape, PDA and prune-lactose agar media. Microsclerotia formation was examined using a dissecting stereo-microscope (WILD M7A, Switzerland) and the light microscope (Leitz as being used throughout, section 2.2.5.). Observations were made based on descriptions made by Isaac (1949), and Hall and Ly (1972). I considered as a microsclerotium a structure that was formed from a single or more hyphae, by repeating budding. Observations were also made at different developmental stages on undisturbed PDA cultures using a light microscope (X10 objective). The lid of the Petri-dish was taken off and the colony of

each isolate was examined microscopically for microsclerotia. Colonies were examined at 0.25 cm, 0.5 cm, 1.5 cm and 2 cm from the hyphal tips.

2.4.1.2. The morphology of conidiophores

Inocula from the margin of a 14-day-old culture on PDA medium were placed on fresh PDA medium and incubated for 10 days in the dark. The lid of the Petri dish was taken off and the undisturbed colony of each isolate was examined microscopically using a X 10 objective (10/0.25, EF 160/-). Observations were based on characters as described by Domsch *et al.* (1980).

2.4.1.3. Comparisons of conidial lengths in haploid (*V.dahliae*) / diploid (*V.d.longisporum*) isolates.

Spore length measurements were made as described in 2.2.5. All isolates in table -2.1b- were tested and the mean conidium length of each isolate was compared with that of 161, a known diploid isolate of *V.dahliae,* from sugarbeet, Sweden, regarded as being *V.d.longisporum* Stark (Jackson & Heale, 1985). The t-test was applied to determine the significance of the means of conidial lengths of grouped population A containing all isolates from different hosts and grouped population B1 containing all proved diploid isolates, as well as the significance of the means of group population B1 and grouped population B2 containing all 'long-spored' isolates from Cruciferous hosts..

2.4.2. Ploidy differences in nuclei of various isolates

Two different fluorochromes(acridine orange and 4,6-dimino-2 phenylindole) that fluoresce when excited with ultra violet light (UV), were used to measure nuclear diameters in the ungerminated conidia for ploidy investigations. In addition, the Feulgen stain (Basic-fuchsin), was employed to measure directly the nuclear stained-DNA content of individual conidia by microdensitometry. For this, ungerminated conidia from a 7-day spread plate (semi-synchronous culture) of each isolate, were used.

2.4.2.1. Nuclear staining

2.4.2.1.1. Acridine orange

The procedure was carried out according to Bainbridge (1971). A spore suspension was prepared by washing a 7-day-old spread plate. The spores were centrifuged for 5 min at 6500 rpm in a MSE microcentrifuge, fixed in 5 % aqueous glutaraldehyde (BDH) v/v for 30 min and washed by 3 consecutive centrifugations in 0.1M sodium phosphate buffer pH: 7.2 [36 ml of 0.2M di-sodium dihydrogen orthophosphate, 14 ml of 0.2M sodium-dihydrogen orthophosphate and 50 ml of distilled water] for 10 min. Finally, the spores were resuspended in 0.1M sodium phosphate buffer containing 100 µg/ml acridine

orange (B.D.H) for 90 min and observed under x1250 epifluorescent conditions using a Leitz Dialux 20EB microscope equipped with Phloemopak 25 fluorescence vertical illuminator supplying UV light from a 50W high pressure mercury lamp. Observations were made using filter block D1, position 2, (exciting filter BP 355-425 nm and suppression filter LP 460 nm).

2.4.2.1.2. DAPI (4,6-diamino-2 phenylindole)

The experiment was carried out according to Hamada & Fugita (1983). A spore suspension was prepared from a 7-day-old spread plate. The spores were centrifuged for 5 min at 6500 rpm in a MSE microcentrifuge (Micro, Centaur, MSE), resuspended in 100% methanol and again centrifuged for 5 min and then washed in Tris buffer 10 mM pH:7.4 , EDTA 10 mM (Ethylendiaminetetraacetic acid, sodium salt) and sodium chloride 100 mM containing 50 ng/ml DAPI (Sigma) for 60 min. Finally, the spores were again centrifuged for 5 min and then resuspended in the same DAPI solution. The stained spores were observed using a Leitz Dialux 20 EB microscope equipped with a high pressure mercury lamp which emitted light at UV<400 nm (filter block D1: exciting filter BP 325-425 nm and suppression filter LP 460 nm). Diameters of the stained nucleus were taken using a calibrating eye-piece micrometer (2.2.5) with a X100 water immersion lens.

2.4.2.1.3. Feulgen

The Feulgen procedure involves fixation, hydrolysis and finally staining. Conidia were harvested with 0.2M sucrose solution from semi-synchronous 7-day-old PDA spread plate cultures with 2 ml of s.d.w. and the suspended conidia collected in two Eppendorf tubes (1 ml in each) and centrifuged for 5 min at 6,500 rpm in a MSE microcentrifuge. The supernatant was decanted and the two spore suspensions were combined in one centrifuge tube ready for fixation. A control strain was used every time viz. isolate 161 (diploid, *V.d.longisporum*), due to the requirement of an internal standard, as staining variation can occur between batches (Dhillon *et al.*, 1977, Jackson & Heale, 1985). To the centrifuge tube was added 0.5 ml of freshly made Helley's fixative (5g mercuric chloride [BDH] and 3g potassium dichromate [BDH] in 100 ml of water and just before use, 1 ml formaldehyde [BDH] was added) and the conidial pellets resuspended and again centrifuged for 10 min at 6,500 rpm in a MSE microcentrifuge. Excessive fixative was removed by resuspention and again centrifugation for 5 min at 6,500 rpm in 0.5M sucrose solution. This was repeated 3-4 times. Cold 5M HCl (BDH) was added to the centrifuge tubes and centrifugation was carried out for 5 min at 6,500 rpm. The acid was decanted and fresh 5M HCl was added to the tubes. The conidia were hydrolysed at 24°C in 5M HCl for 40 min; this relatively 'cold' hydrolysis conditions were found to produce optimum stain density per conidium. The acid was removed by centrifugation as above and the conidia were resuspended in cold 0.5M sucrose and again centrifuged. This was repeated 3 times for 5 min at 6.500 rpm. The supernatant was decanted and 0.5 ml of Feulgen stain solution (Schiff's reagent for detection of aldehydes, Sigma) was added to the tube for 80 min and then centrifuged for 5 min as above. The supernatant was decanted and washed once with SO_2 water (2:1 of potassium metabisulphate [BDH] and 1M hydrochloric acid [BDH]). The supernatant was decanted and 2-4 drops of s.d.w. was added to the

stained pellet of spores, followed by dehydration through a series of alcohol concentrations (10, 30, 50, 70, 90, and 100%).Two to four drops of s.d.w. was added and the stained spore suspension was pippeted onto slides, one drop / slide. The slides with the stained spores were mounted in Euparal, covered each with a coverslip and stored at 23°C for one night for the mountant to set , then stored in the dark at 4 ^{0}C until measurements were taken.

2.4.2.2. Nuclear measurements

2.4.2.2.1. Nuclei in spores stained with acridine orange.

Nuclei stained with acridine orange were not sufficiently clear for their diameter to be measured. For this reason, the stain was used merely in order to confirm that conidia of all isolates of Verticillium under test were uninucleate.

2.4.2.2.2. Measurements of nuclear diameter in spores stained with DAPI

Samples were observed using a Leitz microscope (Dialux 20, E.Leitz Instruments Ltd., Luton, UK) under epifluorescent conditions, with a Fluotar 100/1.20 W objective, using a calibrated eye-piece micrometer (2.2.5.), as used throughout. Measurements were based on an average of 50 readings..

DAPI absorbs UV light (360 nm) and emits blue light. The light given off by fluorochromes, fades (quenches) quite rapidly. DAPI was relatively resistant to fading upon exposure to UV light and continued observations and measurements were made over 20 min time to avoid quenching of DAPI fluorescence at one field of view and then another area on the slide was examined. Also anti-fade mountants like 'Citifluor' could have been used but it was not necessary for this experiment. Correct staining resulted in blue fluorescence for DNA and photographs were taken using a colour Kodak film (ASA 200).

2.4.2.2.3. Measurements of nuclear DNA content by microdensitometry in spores stained with Feulgen

A Vickers M86 Scanning Microdensitometer was used to measure DNA-fuchsin density levels per conidium nucleus. By this scanning system, a number of individual measurements of absorbance (at 560 nm for Feulgen) made regularly over the whole nucleus and each measurement is taken over a sample area, which is referred as a sample size. The accumulated measurements are then summed and integrated by the machine to give a total value of stain density proportional to the amount of DNA-fuchsin present in each nucleus. This relative DNA content (recorded as arbitrary units) was compared to an internal control (on the same slide) of known ploidy , viz. the diploid isolate 161, referred to as *V.d.longisporum* by Jackson & Heale (1985). A X100 oil emersion objective was used and the readings were made with zero background setting (control spot in clear background area) and infinity setting at no illumination. These control settings were checked every five readings to ensure accurate measurements and no background noise. Microdensitometer settings were: Band width control 42 units, wavelength control 57 units related to 560 nm (maximum absorbance for Feulgen stain), spot aperture control S2, gating disc mask A1 and

screen area setting X=1, Y=1 to reduce the noise of the background cytoplasm which can give an appreciable contribution to integrated density readings. Three readings were taken per individual nucleus and the measurements were based on an average of 50 spores sampled randomly. This procedure was repeated for all isolates in table-2.1b-, every time including the internal control (isolate 161, established diploid).

2.4.3. Enzymatic differences between isolates

2.4.3.1. Qualitative observations of polyphenol-oxidase (p.p.o.) and polygalacturonase (p.g.) activity.

2.4.3.1.1. P.p.o. activity

Hyphal tip inocula were taken from 7-day-old PDA cultures and placed in Howell's (1970) medium, modified by Eckerk (1962), incorporating 0.1% tannic acid and incubated for 10 days at 25°C in the dark for indication of extracellular p.p.o. activity. The width and intensity of the dark zones at the periphery of the colony indicated the extent of p.p.o. activity. An absence of dark zone was taken to indicate a complete absence of extracellular p.p.o. activity. Three replicate plates were set up to confirm the results and the experiment was repeated twice.

2.4.3.1.2. P.g. activity

Hyphal tip inocula were taken from 7-day-old PDA cultures and placed in modified Howell's (ibid.) medium incorporating 0.5% polygalacturonic acid (pH 5.0). After incubation for 6 days, the cultures were flooded with 1N HCl for 2 hours to precipitate any polygalacturonic acid left in the medium. The clear zones at the periphery of the colony on the plate indicated their p.g. activity. Three replicate plates were set up and the experiment was repeated twice.

2.4.4. Sensitivity to UV light

The volume of two ml of a suspension of spores washed from a 7 day-old-spread PDA culture, was adjusted to $1X10^6$ spores/ml using the haemocytometer and by appropriate dilution was finally adjusted to 100 spores/ml. One ml of this suspension was pipetted and spread over the entire surface of a Petri-dish with 20 ml of CM and placed below the UV tube. Irradiation was performed in a closed box, with front opening, using a 2537 Å Hanovia bactericidal tube fitted to the ceiling of the box, 20 cm above its base. The lid of the dish was removed and the spore suspension irradiated for up to 180 sec. Plates were assessed for viability 10 days after incubation at 24-25 °C. Three replicates were set up for each treatment.

2.4.5 Randomly amplified polymorphic DNA (RAPD)

2.4.5.1. Mycelium production for DNA extraction

Spore suspensions were prepared in s.d.w. and were used to seed the entire surface of PDA plates. They were incubated for 3 days in the dark giving young colonies and then flooded with 2 ml of liquid complete medium(CM). This was used to inoculate sterile 250 ml flasks (Pyrex, BDH) containing 50 ml liquid complete medium(CM). Flasks were incubated at 25°C for 3 more days on an orbital shaker (SGM-300, Gallenkamp) at 180 r.p.m. in the dark. Hyphal material was harvested on 2 sheets of sterile filter paper (9 cm Whatman, qualitative Number 1) in a Buchner funnel connected to a vacuum line. The filtered mycelium was peeled from the filtered paper and lyophilised for 2 days using a EF4 Modulyo freeze drier (Edward's, Crawly, UK). Samples were ground thoroughly with a pestle and mortar (BDH) and stored at -20 °C until required.

2.4.5.2. Small-scale DNA extraction

DNA was obtained using extraction methods based on those of Raeder & Broda (1986) and Bainbridge *et al.* (1990). Freeze-dried mycelium powder (50 mg) was transferred to an Eppendorf tube with 500 µl of lysis buffer, which comprised:

1M Tris-HCl (Sigma)	100 µl,
NaCl (BDH) 5M	25 µl,
EDTA (ethylene-diamine-tetra-acetic acid, Sigma) 0.5M	25 µl,
SDS (sodium dodecyl-sulphate, Sigma) 20%	12.5 µl,
and s.d.w.	337.5 µl.

The mixture was gently mixed to a homogeneous state with a sterile glass rod by adding 350 µl phenol (BDH) [equilibrated 3 times with 1 vol. 1M Tris-HCl, pH 8.0, and stored under 0.5 vol. 0.1M Tris-HCl, pH 8.0 containing 0.1%(w/v) 8-hydroxyquinoline(BDH)] and 150 µl chloroform(BDH) [prepared as chloroform: isoamyl alcohol(BDH) 24:1] for deproteinisation of the extract. This was then centrifuged at 13,000g for 60 min at room temperature to precipitate proteins in a MSE microcentrifuge(Micro, Centaur, MSE). The upper aqueous face with the nucleic acids was carefully removed and transferred to a fresh Eppendorf tube containing 25 µl RNAase (Sigma) solution (10 mg/ml) and incubated for 30 min at 37 °C to remove RNA. To this extract solution, 1 vol. chloroform was added, mixed gently by inversion and centrifuged at 13,000g in a MSE microcentrifuge for 10 min at room temperature. The supernatant was transferred to a fresh Eppendorf tube and mixed by inversion with 0.54 vol. 100% (v/v) isopropanol (BDH) to precipitate. The DNA precipitated out as a visible clot with gentle tapping. The supernatant was removed and the resuspended pellet was spun down by centrifugation at 4,500g in the MSE microcentrifuge for 5 min. The DNA pellet was rinsed with 70% (v/v) ethanol(BDH), dried under vacuum in a desiccator(TPC 010, Jenscons, Hemel-Hempstead, UK.) for 30 min and was then resuspended at 4 °C

in 100 µl TE(Tris-HCl/EDTA) buffer, pH 8.0, which comprised of :10 mM Tris-HCl, pH 8.0 and 1 mM EDTA , pH 8.0. DNA samples were stored at -20°C until required.

2.4.5.3. Quantification of DNA

The purity and concentration of DNA samples were determined by ultraviolet absorbance spectrophotometric analysis. The amount of ultraviolet radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Readings were taken with a Spectrophotometer (Hewlett, 8452A, Packard Diode Array Spectrophotometer) at 260 nm and 280 nm. An OD_{260} of 1 corresponds to 50 µl/ml of double-stranded DNA, and the ratio OD_{260}/OD_{280} provided an estimation of the purity of the nucleic acids (Sambrook *et al.*, 1989). With a pure sample of DNA, the ratio of OD_{260}/OD_{280} is 1.8. Ratios of less than 1.8 indicate that the preparations were contaminated either with protein or with phenol (Brown, 1993).

2.4.5.4. Genetic fingerprinting by polymerase chain reaction(PCR)

The technique of random amplification of polymorphic DNA (RAPDs) which is a modified polymerace chain reaction (PCR) with single primers for the enzymatic synthesis of DNA was used for strain characterization. This method was previuosly called randomly amplified polymorphic DNA (RAPD) [Williams *et al.*, 1990] or arbitrarily primed polymerase chain reaction (AP-PCR) [Welsh & MacClelland,1990). PCR single short primers (10 or 20 bases) were used at low annealing temperatures (low stringency) to achieve random amplification of genomic DNA. PCR primers are given in table -2.4-.
To 650 µl Eppendorf tubes(Multi-Lube Tube, Bioquote Ltd., Ilkley, UK), the PCR reaction mixture was added which comprised :

16 µl	1.25 mM dNTP's*,
10 µl	10X buffer (supplied by Promega),
6 µl	25 mM $MgCl_2$(supplied by Promega)
66 µl	s.d.w. pH:7,

*[12.5µl of 100 mM dATP, 12.5 µl 100 mM dCTP, 12.5 µl of 100 mM dGTP, 12.5 µl of 100 mM dTTP (Boehringer), and 950 µl of s.d.w. to give a working solution of 1.25 mM]

To this, 1µl of 50 µM primer and 1µl of 100 ng/µl template DNA was added. The amplification was performed in an Hybaid thermal reactor (HBTR1, Hybaid Ltd., Teddington, UK), with the following programme: the template DNA was denatured by heating at 95°C for 5 minutes and with the temperature held at 72°C, 2 units (0.4 µl) of Taq polymerase (Promega) was added and the sample overlaid with 100 µl of sterile light mineral oil (Sigma) to prevent evaporation. The amplification conditions were; annealing at 37°C (for 20mers primers) or 35°C (for 10mers primers) for 2 min, extension at 72°C for 3 min and a denaturing step at 95°C for 1 min to denature the newly synthesized bands. The sequence was repeated for 30 cycles and a final cycle of 2 min annealing followed by 10 min of extension was then performed to ensure completion of the amplification fragments. To ensure that PCR products were amplified genomic

DNA not arising from DNA contamination, 'no DNA' controls were included at every amplification for each primer. PCR products were separated on a 1.5% agarose gels by horizontally gel electrophoresis (Sub-Cell DNA Electrophoresis Cell, Bio-Rad) at 2.5 V cm^{-1} (Procon powerpack NBL) in 0.5% (w/v) Tris-borate (TBE) electrophoresis buffer which comprised :

0.089M	Tris-base (Sigma)
0.089M	Boric acid (BDH)
0002M	EDTA, pH 8.0

and stained for 20 min in 0.5 µg/ml ethidium bromide (BDH). DNA fragments were visualised directly in UV light using UV-transiluminator (UVP TM 20, Genetic Research Instrument Ltd., Dunmow, UK). Photographs were taken using Polaroid film type 667 or 665 (ASA 3000). A 1kb 'ladder' was used as marker to determine the fragment sizes of PCR products.

Table -2.4-.Base sequence and origin of PCR primers

PRIMER	BASE SEQUENCE	ORIGIN
P2[1]	5' CACCGCCCCAAAATGGCCAC 3'	*Penicillium hordei* IGS* region
P4a[1]	5' CACGTACAGTTCGCTCGTCG 3'	*Penicillium hordei* IGS* region
P4[1]	5' TACACCTCCC 3'	*Penicillium hordei* IGS* region
P6[1]	5' GTCCTCAGTCCCCCAATCCC 3'	*Penicillium hordei* IGS* region
P5a[1]	5' GAGTAGGACTACTGCAGGAG 3'	*Penicillium hordei* IGS* region
0PA-1[2]	5' CAGGCCCTTC 3'	OPERON synthetic oligonucleotide
OPA-2[2]	5' TGCCGAGCTG 3'	OPERON synthetic oligonucleotide
0PA-5[2]	5' AGGGGTCTTG 3'	OPERON synthetic oligonucleotide
OPA-6[2]	5' GGTCCCTGAC 3'	OPERON synthetic oligonucleotide
OPA-7[2]	5' GAAACGGGTG 3'	OPERON synthetic oligonucleotide
OPA-8[2]	5' GTGACGTAGG 3'	OPERON synthetic oligonucleotide
OPA-9[2]	5' GGGTAACGCC 3'	OPERON synthetic oligonucleotide
OPA-10[2]	5' GTGATCGCAG 3'	OPERON synthetic oligonucleotide
0PA-13[2]	5' CAGCACCCAC 3'	OPERON synthetic oligonucleotide
OPC-2[2]	5' GTGAGGCGTC 3'	OPERON synthetic oligonucleotide
OPC-3[2]	5' GGGGGTCTTT 3'	OPERON synthetic oligonucleotide
0PC-8[2]	5'TGGACCGGTG 3'	OPERON synthetic oligonucleotide
0PC-9[2]	5' CTCACCGTCC 3'	OPERON synthetic oligonucleotide
OPC-11[2]	5' AAAGCTGCGG 3'	OPERON synthetic oligonucleotide
OPC-15[2]	5' GACGGATCAG 3'	OPERON synthetic oligonucleotide
OPC-19[2]	5' GTTGCCAGCC 3'	OPERON synthetic oligonucleotide

2.4.5.5. Cluster analysis of RAPDs data

The estimation of the sizes of the DNA fragments generated by RAPDs separated on each gel was calculated by constructing calibration curves based on the mobilities of DNA size markers (GIBCO BRL 1 kb ladder). The data from generated fragments from 3 different primers were converted as follows: 1 if a

[1] PCR primers (supplied by Dr. T.Roberts, 1992, KCL) were based on conserved sequences of the rDNA IGS region from *P.hordei.*

[2] PCR primers were supplied by OPERON technologies. Sequences were selected radomly, with the requirement that the (G+C) content is 60% to 70% and that they have self-complementary ends. These primers were sold in kits of 20 sequences each and are designed Kit A through Kit AN.

specific molecular weight band was present and 0 if it was not present and were analysed using an average-linkage cluster analysis (unweighted pair group method with arithmetic averages, UPGMA analysis), with Jaccard's coefficient (Austin & Priest, 1986) that discounts negative matches. The analysis was performed using the numerical taxonomy and multivariate analysis system (NTSYS-PC Version 1.8 software package, [Rohlf,1988]). Results from cluster analysis were presented as a dendrogram to display the genetic distance between strains (Sneath & Socal, 1973). Also the data were analysed by co-ordination analysis using the same software that generated a three dimensional output of the principle groups.

2.4.6. Chromosome-length DNA polymorphisms

The aim was to determine the level of chromosome-length DNA polymorphisms among diploid isolates of the strain *V.d.longispogum* as compared with haploid isolates of 'normal' haploid strains of *Verticillium dahliae.* This was done by separating chromosomes by pulsed field gel electrophoresis (PFGE) in a contour-clamped homogenous electric field that alternated between two orientations of 120°. This system, designated CHEF (Chu *et.al.*,1986) permits resolution of high molecular weight DNA (more than 20kb) in straight tracks. Each DNA molecule in the gel has to reorient with every change in the field direction. Large DNA molecules require more time to reorient than short DNA molecules and so short molecules progress faster than bigger ones to the bottom of the gel.

For this procedure, chromosome-length DNA was extracted under gentle conditions from hyphal protoplasts.

2.4.6.1. Mycelium production for protoplast isolation

A conidial suspension was obtained from a 7-day-old spread-culture in s.d.w. and was used to inoculate 250 ml flasks (Pyrex, BDH) containing 50 ml liquid potato dextrose broth. The flasks were incubated at 25 °C on an orbital shaker (SGM-300, Gallenkamp) for 48 hours at 180 r.p.m. in the dark (24 hours was found to be insufficient for mycelia production). Mycelia were harvested aseptically by vacuum filtration using a sintered glass filter n°2 (BDH) and rinsed twice with Ringer's solution, followed by a rinse with sodium maleate buffer pH 5.2 with 0.7 M NaCl (SM-NaCl). The SM-NaCl buffer was prepare by adding :
125 ml solution A : 11.607 g maleic acid and 4 g NaOH in 500 ml of distilled water,
52 ml solution B : 4 g NaOH in 500 ml of distilled water and 20.454 g NaCl.

Mycelia (100 mg/ml of lysing enzyme mixture) were digested with lysing enzyme mixture in SM-NaCl buffer which comprised:
5 mg/ml Driselase (Sigma) and
10 mg/ml Lysing enzymes (Sigma)

The lysing preparations were incubated in a Petri-dish with constant agitation at 60 r.p.m at 32°C for 2.5 hours in an incubator (HYBAID). The formation of protoplasts was checked every half hour by taking a sample and observing under the light microscope (Leitz). The protoplasts were separated from the non-digested mycelia by filtration(sintered glass n°1), washed with SM-NaCl buffer and concentrated by

centrifugation at 800 r.p.m. for 15 min in a centrifuge (MSE Chilspin 2, FISONS). The number of protoplasts were adjusted to $2X10^6$ protoplasts / ml using a haemocytometer (2.2.4.).

2.4.6.2. Extraction of chromosomal-length DNA

Large molecules of DNA are very fragile and they can be broken easily during extraction. To prevent breakage, intact protoplasts embedded in low melting agarose were lysed and deproteinized *in situ*, as agarose plugs.

The pelleted protoplasts were mixed in 1.3 % w/v aqueous solution of preparative grade, molten low melting agarose (Bio-Rad) 1:1(v/v) at 45 °C to give a final concentration of $1x10^6$ protoplasts / ml. Agarose plugs were prepared by pouring the protoplast agarose suspension in Biorad's sample mould which was then allowed to cool in an ice bath for 10 min. The sample mould produced 20 X 9 X 12 mm agarose blocks. This block thickness allowed efficient diffusion of enzymes for lysis and deproteinization and also permitted samples to be loaded into wells formed with Bio-Rad's standard, well-forming cobs. The agarose plugs were incubated in a solution of 2 mg/ml proteinase K (Sigma) to degrade DNA binding and other proteins, for 48 hours at 50°C in NDS buffer which comprised :

0.5M	EDTA, pH 8.0, (to ihibit nuclease activity),
10 mM	Tris-HCl, pH 9.5 and
1% w/v	sodium lauroysarcosinate (to destroy the integrity of the nuclear and cytoplasmic membranes),

followed by three, 30 min washing steps at 50°C in 50 mM EDTA (pH 8.0) to remove all the cellular components contaminating the DNA. Plugs were stored at 4 °C in 50 mM EDTA (pH 8.0), Vollrath and Davis (1987), until required (for at least one year).

2.4.6.3. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis(PFGE) was performed with the contour-clamped homogenous electric field system, field angle 120° (CHEF DR-II, Bio-Rad), by using 0.5 X TBE as a running buffer [0.089 M Tris-Borate, 0.089 M Boric acid and 0.002 M EDTA] (Maniatis *et al., 1982*). DNA plug inserts (processed according to 2.4.6.2.) were loaded into sample wells of an 0.8% agarose gel and sealed with low-melting agarose at the same concentration (Migheli *et.al.*, 1993). Electrophoresis conditions were : 3600s for 48 h, 3100 s for 48h, 2600s for 48 hours, 2100 s for 48 h, 1600 s for 24 h, 1100 s for 24 h and 1000s for 24 h. The field strength was 50V and the buffer temperature was maintained at 4°C throughout the experiment.

2.4.6.4. Estimation of chromosome sizes

After electrophoresis, gels were stained in ethidium bromide (0.5 µg /ml) for 30 min and destained with several changes of tap water at 4°C. The three chromosomes (3.5, 4.6 and 5.7 Mb) of *Schizosaccharomyces pompe* strain (Bio-Rad) were used as molecular size standards to construct calibration curves.

2.5. Host-pathogen interactions. The role of the myrosinase-glucosinolate system in the defence mechanism of oilseed rape against *V.dahliae.*

To investigate the role of myrosinase measurements were conducted for myrosinase activity from infected and non-infected control plants of oilseed rape (cultivar Cobra) inoculated independently with an avirulent isolate of a non-pathogenic strain of *V.dahliae*, tomato, USA, (130, haploid) and a virulent isolate of a pathogenic strain *V.d.longisporum*, sugarbeet, Sweden, (161, diploid). The enzyme was localized *in situ* by immunogold-labelling in tissues of infected and non-infected plants at different times after root inoculation (root dipping).

To investigate the possible role of glucosinolates, these compounds were extracted from infected and non-infected plants and analysed after separation with high performance liquid chromatography (HPLC).

2.5.1. Myrosinase assays

Myrosinase assays were carried out with infected and non-infected oilseed rape seedlings using a modified method as described by James & Rossiter (1991). The seedlings of oilseed rape cultivar Cobra (grown in the greenhouse) were inoculated 2 weeks after sowing with a spore suspension containing $1X10^6$ spores/ml of either a virulent isolate of *V.d.longisporum* (161) or an avirulent isolate of *V.dahliae* (130) by the root-dipping method.

2.5.1.1. Extraction of crude soluble myrosinase

The tissues (roots, stems, cotyledons and leaves) of inoculated and non-inoculated seedlings of oilseed rape (cultivar Cobra) were cut 3, 10 and 15 days after inoculation and freezed-dried for 24 hours. They were extracted in 10 mM imidazole-HCl buffer pH 6.2 (Sigma), [100 mM stock: 85.5 ml 1M HCl and 250 ml 0.4 M imidazole] with 10 µg/ml leupeptine (20µl) [Sigma] at 4 °C by grinding in a mortar with a small amount of washed sand. The homogenate was centrifuged for 5 min at 13.000 g in a microcentrifuge. The non-soluble material was discarded and the supernatant was dialysed against the same buffer with dialysis tubing (Sigma D-9777) at 4°C for 24 h. The dialysis tubing retained most proteins of MW >12.000 and was pre-treated by boiling in water, washing in fresh water and storage at 4 °C until required.

2.5.1.2. Extraction of microsomal myrosinase

The roots of 10 oilseed rape seedlings were cut 4 days after inoculation for each treatment and extracted with 0.1M Phosphate buffer pH:7.4, 0.25M sucrose, 5 mM EDTA, 10 mM 2-ME (mercaptoethanol) and 1% BSA (Sigma) and 10 µg /ml leupeptin at 4 °C by grinding in a pestle and mortar with a small amount of washed sand. The homogenate was centrifuged down at 20,000 g for 20 min, filtered and resuspended at 100,000 g for 1h. The pellet (microsomal) was taken carefully and dissolved in resuspension buffer (0.1 M Phosphate pH 7.4, 30% glycerol and 1 mM 2-ME).

2.5.1.3. Purification of soluble myrosinase (supernatant)

The soluble enzyme was desalted by passage through a PD-10 column (Pharmacia) equilibrated in 50 mM sodium acetate buffer pH 5.5.

2.5.1.4. Protein estimation in myrosinase samples

The protein content of the sample (desalted and non-desalted) was determined by Biorad-Protein assay. 200µl of Biorad Dye Reagent were mixed with 800 µl of the myrosinase sample, mixed by gentle inversion of the test tube and the mixture left for 10 min. The absorbance was measured against a blank (sample buffer) at 595 nm spectrophotometrically. Protein estimations were made from plotted standard curves OD 595 versus standard concentrations of BSA (0-25 µg/ml). BSA in 50 mM sodium acetate, or 0.1 M phosphate buffer gave a good linear absorbency when a standard curve was plotted.

2.5.1.5. Estimation of myrosinase activity

An assay based on the determination of glucose released on the hydrolysis of sinigrin by myrosinase was used to determine the enzyme activity in inoculated and uninoculated oilseed rape seedling tissues.

The assays were conducted in a final volume of 900 µl, comprised of sinigrin (20 mg/ml), ascorbic acid (10 mM) and (i) desalted enzyme solution buffered in 50 mM sodium acetate, pH 5.5, or (ii) microsomal enzyme buffered in 0.1 M phosphate. Three different enzyme volumes were used for each assay since the exact concentration of the enzyme in the extract could not be known initially (see Table -2.5-).

Table -2.5- Myrosinase assays

ENZYME	SINIGRINE	ASCORBATE	BUFFER	FINAL VOLUME
100 µl	180 µl	30 µl	590 µl	900 µl
200 µl	180 µl	30 µl	490 µl	900 µl
300 µl	180 µl	30 µl	390 µl	900 µl

Assay vials with desalted soluble enzyme were incubated for 40 min at 35 °C in a water bath. Assays with the non-desalted enzyme were carried out without ascorbate in a final volume of 900 µl and incubated for 3 hours at 35 °C water bath. The reaction was stopped by boiling. Aliquots of the mixture were (100µl) then removed, boiled for 3 min to inactivate the enzyme and then analysed for their glucose content with an enzymatic (hexokinase-based) glucose test kit (Sigma). To each test tube 20 µl of speciment was added with 1ml glucose assay reagent, mixed gently by swirling and allowed the mixture to stand 5-10 min and then to each tube 100µl of 1M HCl acid was added. A spectrophotometric assay was used to determine the activity of the purified enzyme at 520 nm using inactivated enzyme as blank. The

readings were completed within 30 min and glucose concentrations were calculated using the glucose standard that was supplied in the Sigma glucose kit (1 mg/ml).

2.5.2. Localization of myrosinase

2.5.2.1. Histochemical localization of myrosinase in hand-cut sections of radicles

Seeds of oilseed rape (*Brassica napus*, cultivar Pasha) and *Raphanus sativus* were germinated in Petri-dishes with sterile moist filter paper (Whatman 9 cm No 1) at room temperatures subject to normal light variation for varying periods. Hand-cut sections of the radicles were taken 4h, 24h, 48h, 72h and 168h after imbibition and incubated for 1h in a solution of 5 mg/ml sinigrin, 10 mg/ml barium chloride and 0.5 mg/ml L-ascorbic acid (Sigma) in distilled water (which resulted in dense deposits of barium sulphate if myrosinase was present), as described by Phelan *et al.* (1984). Controls were incubated into the same solution without sinigrin. The hand-cut sections were stained in lactophenol cotton blue for 1h and then transferred into pure lactophenol. Samples were observed immediately under the light microscope (Leitz) with a X50 objective to find myrosine cells localized by their barium sulphate deposits (black).

2.5.2.2. Immunocytochemical localization of myrosinase

Immunolabelling is a method of localizing a protein of interest (e.g. myrosinase) using an antibody raised against an antigenic determinant of that protein. Immunocytochemical localization of myrosinase was achieved by using two different antibodies raised against myrosinase.

The principle of immunolabelling is that specific primary antibodies recognise antigen sites (i.e. myrosinase), exposed at the cut surface of tissue sections and bound to them. Colloidal-gold particles (used as electron dense markers) conjugated to secondary antibodies which attach to the bound primary antibody sites are then visualised in the electron microscope. Non-specific binding is preventing by blocking non-antigen sites with blocking agents such as bovine serum albumin (BSA), normal goat serum and Tween-20.

2.5.2.2.1. Tissue material used for immunogold localization studies

To localize the enzyme *in situ*, in preliminary tests radicles and cotyledons of germinated oilseed rape seedlings from seeds that had been imbibed in water for a few hours, were examined in order to investigate myrosinase. The enzyme is localized in specialized cells named myrosin cells in the peripheral cortex region of the radicles (Thangstad *et al.* 1990,1991; Bones *et al.*, 1991).

For host/pathogen studies, oilseed rape (cultivar Cobra) seeds were sterilised with 0.5% (v/v) sodium hypochlorite and grown on wet s.d.w. filter paper in Petri dishes for 3 days and were then transferred to vermiculite for a further 11 days during which time they were fed with half-strength Phostrogen. Seedlings were grown in the greenhouse under semi-controlled conditions (2.3.2.) at 18°-25°C and allowed 16 hours of light and 8 hours of dark per day.

The roots of 14-day old oilseed rape seedlings were dipped for 15 min in a spore suspension ($1X10^6$ spores/ml) of either a virulent isolate of the pathogenic strain *V.d.longisporum*, sugarbeet, Sweden, (161, diploid) or a non-virulent isolate of the non-pathogenic strain *V.dahliae*, tomato, USA,(130,haploid). In the control, roots of 14-day-old seedlings were dipped in s.d.w. water for 15 min.

All treated seedlings were repotted in vermiculite and after a further 3, 10,15 and 20 days, the roots, cotyledons hypocotyls and leaves in each treatment were prepared for electron microscopy studies as follows. Cut-stem inoculations were also perfomed. Fifteen days-old plants were cut at the base of hypocotyl and dipped in spore suspension ($1X10^6$ spores/ml) of either the virulent isolate (161) of the diploid strain *V.d.longisporum* or the avirulent isolate (130) of the haploid strain *V.dahliae* (tomato) for 45 minutes.

2.5.2.2.2. Preparation of specimens for electron microscopy

2.5.2.2.2.1. Fixation

Two different chemical fixations were compared to determine the conditions which provided the best preservation of the tissue viz.:

-(a) 2.5% glutaraldehyde (Agar Scientific) in 0.05M cacodylate buffer (4.28g sodium cacodylate [Agar Scientific] in 150 ml of d.w. to make 0.1M buffered with 1M HCl) (Sabatini *et al.*, 1963) at pH 7.2 for 16 hours at 4°C,

-(b) 2% glutaraldehyde and 4% paraformaldehyde in 50 mM phosphate buffer(36 ml of O.2M Na_2HPO_4 $2H_20$ and 14 ml 0.2M NaH_2PO_4 H_2O in 50 ml of d.w.) at pH 7.2 for 7 hours (Thangstad *et al.*, 1991). This was prepared by warming the paraformaldehyde in the buffer at 40 ^{0}C for 5 h until dissolved, cooled down in the fridge and then the glutaraldehyde was added.

Samples of each treatment were cut into 1 mm^3 pieces or cubes on a sheet of dental wax in a fume cupboard with a new, single-edged, razor blade (Boots Chemist Department) whilst bathed in the fixative. Great care was required during cutting to ensure that tissue damage was minimised to prevent autolysis occurring as a result of cell injury. As soon as the pieces were prepared they were transferred to pre-labelled glass vials (containing the fixative) using a wide-mouthed Pasteur pipette. The fixative was at least 10 times greater in volume than the specimen. After primary fixation (16h or 7h) the fixative was removed with a fine glass Pasteur pipette (Volac Disposable Glass Pasteur pipette 230 mm, John Poulten Ltd) in the fume cupboard. The fixative was replaced with the same buffer as that used for the fixative and the procedure repeated three times, each for 45 min. Half of the specimens from both uninfected and infected tissue underwent post-fixation with 1% osmium tetroxide in 0.05 M cacodylate buffer for 1 h.

2.5.2.2.2.2. Dehydration

Dehydration was accomplished by passing the fixed specimens through a graded series of solutions of increasing concentrations of ethanol. The specimens remained in the glass vials in which they were fixed throughout dehydration. One solution was removed carefully with a Pasteur pipette and the next poured in

with care to ensure that the specimen was retained in the solution and that the volume of the solution was much greater than the volume of the specimen. The schedule used for dehydration was 30, 45, 50, 70, 90 and 100% (with 3 changes) v/v ethanol for 30 min. The 100% dehydrating agents were soaked with a molecular sieve of potassium alumina silicon (3 AMP) before use to remove any residual water.

2.5.2.2.2.3. Infiltration.

After fixation and dehydration the samples were infiltrated with LR white resin for immunogold labelling and for conventional staining. The time suggested for infiltration of the LR white resin by the manufacturer was 24 hours. This time was not sufficient for oilseed rape tissue, giving poor quality ultra-thin sections which were very fragile under the electron beam. Therefore, infiltration was performed over a period of 4 d, changing the resin every 24 h. Lids were left off after each change to allow excess solvent to escape. After the last change the specimens were blocked out in gelatine capsules(Agar Scientific) which were closed to expel any air as LR white requires anaerobic conditions to polymerize properly) and put in a 60°C oven for 48 h to allow polymerization of the resin. LR white was very convenient to handle and gave high quality, thin and ultra-thin sections.

2.5.2.2.2.4. Sectioning.

2.5.2.2.2.4a. For light microscopy.

The block resin containing the tissue was first 'trimmed' with a razor blade. One-two µm sections of tissue were cut from the resin blocks on a ultramicrotome (Reichert, OMU3) with glass knives made with a knife maker (LKB, Type 7800B, Stockholm), collected on glass slides and viewed after staining with 1% Toluidine blue in saturated borax for approximately 30 sec on a 60°C hot plate, under a light microscope. The tissue was examined for myrosine cells and fungal spores or hyphae and when satisfied with the area viewed the area was selected and 'trimmed down' for further investigation with the electron microscope.

2.5.2.2.2.4b. Sectioning for electron microscopy

The resin block face with the selected area of tissue was carefully re-trimmed down to a trapezium shaped (block face approximately 0.5 mm across) and 100 nm ultra-thin sections were cut with a high quality glass, or diamond, knife. These sections were finally collected on chloroform-cleaned, 300 mesh uncoated copper grids (Agar Aids), or 100 mesh formvar-coated, copper grids for conventional staining of membranes and structures within the plant cells. Formvar-coated grids were used to provide a larger area of section without masking by grid bars. Alternatively, sections were mounted on 300 mesh gold grids (Agar Aids) for immunogold labelling of myrosinase. All sections tended to compress after sectioning, and so were stretched out on the grids by passing the grids for a few seconds through a flamed loop.

2.5.2.2.3. Production and purification of 2 different myrosinase antibodies

2.5.2.2.3.1.Production

Two different antibodies were used to localize the enzyme *in situ*. The first was raised against myrosinase I by Dr. D. James, Wye College, University of London and the second was raised against myrosinase by Prof. A. Bones, Unigen, Center for Molecular Biology, University of Trondheim, Trondheim, Norway. The first polyclonal antibodies against myrosinase I, named M1, from *Brassica napus* were elicited in a New Zealand white rabbit by 2 subcutaneous injections of 100 µl of highly-purified protein in Freud's adjutant to make the protein more immunogenic. Injections were 2 weeks apart and anti-serum was collected 2 weeks after the final injection (James & Rossiter, 1991). The second antibody named K089 had been raised against myrosinase extracted from *Sinapis alba* as described in Thangstad *et al.* (1991).

2.5.2.2.3.2. Purification

The first antibody (M1) was purified by the present author using a standard rabbit IgG purification procedure involving the technique of affinity chromatography. This was carried out using the Econo-Pac protein A kit (Bio-Rad) which contained a protein A column with 2 ml of Affi-Gel protein. This protein A agarose consists of purified protein A coupled to cross-linked agarose beads via chemically stable amide bonds.

The purification procedure involved three steps. A sample preparation step using the Econo-Pac 10 PG column, a purification step using the Econo-Pac protein A column and a buffer exchange step using a fresh Econo-Pac 10 PG column. All steps were carried out according to instruction manual booklet supplied by Biorad in the kit.

The 10DG column was equilibrated with 20 ml of binding buffer[1] (B.B.) and 3 ml of the rabbit serum was added to the column. The first 3 ml eluted were discarded and 4 ml of B.B. was added to elute the serum, while 4 ml fraction was collected from the column. The protein A column was equilibrated with 10 ml of binding buffer and then 2 ml of the previously eluted 4 ml fraction of rabbit serum was run through the column. The column was washed with 20 ml of binding buffer and IgG eluted with 10 ml of elution buffer[2] (E.B.). A further 20 ml of the E.B. was run through the column to ensure that all the IgG had been removed. The eluted fraction of IgG was neutralised, desalted and buffer exchanged immediately using a new 10DG column. Three ml of the IgG-contain fraction was applied to this new 10DG column, discarded the first 3 ml eluted and 4 ml of phosphate saline buffer (PBS) pH 7.4 which comprised:

8g	NaCl,
0.2g	KCl,
1.44g	Na_2HPO_4,
0.24g	KH_2PO_4 and
1000 ml	s.d.w.

[1] The binding buffer was supplied as a pownder and reconstituted according to manual instructions.
[2] The elution buffer was supplied as a powder and reconstituted according to manual instractions.

was added and 4 ml fraction was collected. This fraction contained the protein A purified IgG. Finally the protein A column was washed with 10 ml of regeneration buffer supplied by Econo-Pac protein A kit and stored in buffered PBS containing 0.05% sodium azide (NaN_3) as antimicrobial agent at 4°C. The final eluted fraction was approximately a 1 in 10 dilution.

2.5.2.2.4. Staining of ultra-thin sections for electron microscopic observations

2.5.2.2.4.1. Immunogold labelling

This technique involves blocking, incubation in the primary antibodies (M1 or K089), incubation in the secondary antibody-gold and finally, staining.

For this, a humid chamber was set up by placing a parafilm-covered slab of dental wax (divided into the appropriate strips) on a saturated piece of blue paper inside an upturned plastic box. Grids supporting plant tissue were individually transferred into drops (20µl) of reagents, tissue face-up, using a fine pair of tweezers or were individually placed on the top of drop meniscus of reagents, tissue face down, using a loop. By using this procedure, tissue was not damaged so much by the consequent transfers in the reagents. Two different procedures were used:

Procedure -1-

Reagents

Washing buffer A (WB-A) consisting of :

20 mM	TBS (2,42g Tris and 29,20g NaCl)
0.1% (w/v)	BSA (Sigma),
0.02%(w/v)	Sodium azide and
0.05%	Tween 20, pH 7.4 ,

Blocking buffer A (BB-A) consisted of :

2% (w/v)	BSA solution in TBS.

Primary antibody

A suitable concentration of the purified primary antibodies to the myrosinase was found to be 1/1000 in WB-A for M1 antibody and 1/15.000 in WB-A for K089 antibody.

Secondary antibody

A goat anti-rabbit IgG conjugated with gold was prepared in 1/100 V/V dilution in WB-A. Three different secondary antibodies were used. One was available from Biocell research laboratories, the other from Sigma and the third from Amersham.

Table-2.5a-Summary of steps (Procedure -1-)

STEP	REAGENTS/PROCEDURE	TIME	CONDITIONS
1.Blocking	Grids were placed in 20 µl of WB-A	60 min	Room t
2.Incubation	Grids were incubated in 20 µl of primary antibody	16 h	4 ^{0}C
3.Rinse	a. Grids were placed into 20 µl of WB-A and stringently washed in a flow of the same buffer, while still held in tweezers and after 30 sec were placed in a fresh drop of WB-A. b. Grids were placed on the top of drops of WB-A and passed through series of drops of the same buffer while excess buffer was removed with filter paper.	30 sec	Room t
4.Incubation	Grids were incubated in 20 µl of secondary antibody solution	60 min	Room t

Grids were individually placed in each strip, and the 300 mesh, uncoated gold grids supporting plant sections were individually placed into drops, initially wet with WB-A to allow easier penetration through the drop meniscus.

Procedure -2-

Reagents

Blocking buffer B (BB-B) consisted of :

5% (v/v) goat non-immune serum,
0.3% (v/v) Tween 20,
1% (w/v) BSA,
0.5% (w/v) non-fat dry milk and
0.02% (w/v) sodium azide
in 20 mM TBS, pH 7.4.

Washing buffer (WB-B) consisted of :

1% (v/v) normal goat serum
0.05% (v/v) Tween-20
0.1% (w/v) BSA
0.02% (w/v) NaAzide
in 20 mM TBS buffer pH 7.4
Primary antibody, Secondary antibody
As in procedure-1-

Table-2.5b-Summary of steps (Procedure -2-).

STEP	REAGENT/PROCEDURE	TIME	CONDITIONS
1. Blocking	Grids were placed in 20 µl drops of BB-B.	15 min	Room t
2.Incubation	Grids were incubated in primary antibody diluted in WB-B.	30 min	Room t
3. Rinse	As in procedure-1- with WB-B	30 sec	Room t
4. Incubation	Grids were incubated in a drop of secondary antibody.	60 min	Room t

After step 4, (in both procedures),the grids were again thoroughly washed in washing buffer and twice in TBS for 5 min. After the last wash in TBS, they were post-fixed in 2% glutaraldehyde in TBS for 5 min and washed again in distilled water [2 X 5 min] (Thangstad *et.al.*,1991). The sections were contrasted with 1% aqueous osmium tetroxide for 15 min and washed, followed by 2% aqueous uranyl acetate for 10 min and Reynolds lead citrate (Reynolds, 1963) for 3 min. The post-embedding, on-grid osmium staining was performed as described by Berryman & Rodewald (1990). All steps were carried out at room temperature . Controls were set up to test the specificity of the primary antibodies. The anti-myrosinase primary antibody (M1) was saturated with purified myrosinase protein before incubating the plant sections in it. Also, a rabbit anti-goat was used as a secondary antibody.

2.5.2.2.4.2. Conventional staining

Plant sections on 100 mesh copper grids were stained with 2% aqueous uranyl acetate for 10 min in the dark and Reynolds lead citrate for 3 min. Care was taken to ensure that no lead carbonate deposits were in the lead citrate solution before it was used. Lead carbonate deposits are formed upon exposure to air (Hayat, 1989). Grids were washed thoroughly with d.w. in between stains. They were dried thoroughly under an electric lamp while still held in tweezers and viewed under the electron microscope (Hitachi, H-7000, Electron Microscope).

2.5.3. Immunoblot analysis of myrosinase using anti-myrosinase polyclonal antibodies (MI, KO89)

2.5.3.1. Gel electrophoresis for protein separation

2.5.3.2. Preparation of oilseed rape plant material

For SDS -PAGE (sodium dodecyl-sulphate-polyacrylamide gel electrophoresis) separations, crude soluble protein was obtained as in section 2.5.1.1. The protein samples were treated with sample buffer (SDS reducing buffer), 1:4 v/v, which consisted of:

4 ml s.d.w.,

1.0 ml	0.5 M Tris-HCl, pH 6.8,
0.8 ml	Glycerol
1.6 ml 10% (w/v)	SDS
0.4 ml	2-ß-mercaptoethanol
0.2 ml 0.05% (w/v)	bromophenol blue-tracking dye

and boiled at 95 ^{0}C for 4 min to denature the proteins to allow them to bind with SDS during electrophoresis. These polypeptides-SDS complexes migrate according to their polypeptide size (Laemmli, 1970).

2.5.3.3. Separation of protein

Polypeptides were resolved in 12% (w/v) acrylamide vertical mini slab gels (Mini-PROTEAN II dual slab cell, Bio-Rad) according to the procedure of Laemmli, (1970). Two discontinuous (Laemmli) polyacrilamide gels consisting of a resolving/separating gel (lower) and a stacking gel (upper) were set up and samples, after treated with SDS reducing buffer, (2.5.3.1.), were loaded in gel (20 µg of protein per track). Reagents and the SDS gel were prepared according to the manufacturer's instructions (Biorad Mini-Protean II dual slab cell instruction manual). A multicoloured 'rainbow weight markers' was used to ensure full transfer of polypeptides onto the nitro-cellulose filter. The gels were run at a constant voltage settings of 200 volts for 45 min; at this time the polypeptide compounds were then completely separated in the resolving gel. They were then removed and one was stained in Coomassie Blue and the other Western-blotted onto nitro-cellulose according to the procedure of Burnette (1981) and an immunoblot carried out. The MW of myrosinase was calculated by comparing its electrophoretic mobility with those of standard markers (Sigma high molecular weight markers).

2.5.3.4. Staining of the gel

After SDS-PAGE separation, polypeptides were stained with 0.1% Coomassie Blue R-250 in fixative (40 % ethanol and 10 % acetic acid) for 30 min and destained in destain 1 (40 % ethanol and 10 % acetic acid) for 30 min so as to remove background stain. Finally, the gel was destained in destain 2 (5% ethanol and 7% acetic acid) overnight.

2.5.3.5. Western (immunological) blotting

2.5.3.5.1. Electrophoretic blotting

Polypeptides were transferred electrophoretically (transblotted) from SDS on to a nitro-cellulose filter (S2S BA 83, 0.2 µm 9X7 cm) using the Trans-Blot® (BIORAD) and this nitro-cellulose was immunoblotted to test the specificity of the purified antibody against myrosinase I, M1. A 'sandwich' was prepared with the following successive layers: A polyethylene sheet (Bel-Art) 13.5X14 cm and 1.6 mm thick; three thicknesses of filter paper 9S & grade 470) 13.5X10 cm; the SDS polyacrilamide slab gel (13.5X9.5 cm with the stacking gel removed: a nitro-cellulose sheet (S&S BA 83, 0.2 µm) cut to the size of the gel:

three more thickness of filter paper and finally, another sheet of porous polyethylene. The so called "gel sandwich' was enclosed within the carbon electrodes and had a semi-dry blot effect. The current was set up at a constant 189 MA and run at maximum voltage. Gradually the current was increased to 190 mA and left for 30 min to allow successful transblotting of the polypeptides onto the nitro-cellulose.

2.5.3.5.2. Immunodetection.

The nitro-cellulose was placed in TBS for 5 min and then cut and the transblotted markers placed in amino black for 2 min. The nitro-cellulose with the transblotted polypeptides were immersed into TBS for 5 min followed by 90 min in blocking solution which comprised:

5%	non-fat dry milk in TBST, pH 7.4
TBST was consisted:	
20mM	Tris-HCl
0.5M	NaCl
0.1%	BSA
1/2 ml of 0.05%	Tween-20.

This was gently agitated using an orbital platform at room temperature. The nitro-cellulose was washed in TBST for 5 min and the nitro-cellulose was incubated in the primary antibody (1/2000 dilution for M1 and 1/10000 dilution for K089) in a water bath at 25 ^{0}C for 26 hours overnight. The blocking solution was then decanted, washed in TBST for 5 min to remove any unbound primary antibody, and incubated with secondary antibodies (purified goat-anti-rabbit IgG [H+L] alkaline phosphatase conjugate[1] at a dilution of 1/3000 [incubation time : one hour] or ECL [enhanced chemiluminescence] horse radish peroxidase labelled antibodies [Bio-Rad]), to detect the protein-antibody complexes. All these steps were performed according to manufacturers instructions. The nitro-cellulose membrane was then dried on filter paper and kept in the dark until later photographed.

2.5.4. Analysis of individual glucosinolates

2.5.4.1.Sample collection for glucosinolate analysis

Oilseed rape seedlings were inoculated (root-dipping) at 14 days as described above (2.5.2.2.1) with either a virulent isolate (161) of the diploid strain viz. *V.d.longisporum* or an avirulent isolate (130) of a haploid strain of *V.dahliae* and investigated at 3, 10 and 15 days after inoculation. Non-inoculated plants were used as controls. Twenty replicate plants were used for each treatment and the experiment was repeated twice. Roots, hypocotyls, cotyledons and leaves were sampled separately. Samples were collected, their fresh weight recorded and put in freezer (-71°C) for 4 h after which they were lyophilised overnight. Samples were kept in a desiccating box at -20°C until required.

[1] This was provided in the Bio-Rad Immuno Blot assay kit.

2.5.4.2.Preparation of reagents for the analysis of glucosinolates

2.5.4.2.1.Buffers

0.02 M Acetate buffer

1.2 ml	Acetic acid
990 ml	Water

This was buffered at pH 5.0 with NaOH and made up in 1 l

1 M Sodium Acetate

136.08 g	Sodium acetate

in 1 l of distilled water.

2.5.4.2.2.Sephadex gels.

DEAE-Sephadex A25 suspension

DEAE-Sephadex A25 (Sigma) was soaked in 0.02 M acetate buffer and stirred overnight (10 g in 400 ml of 0.2 M acetate buffer). The next day the upper face was discarded, water added and then it was left overnight to settle. After discarding the upper face, 0.02 M acetate buffer was added.

DEAE Sephadex A25 (acetate form).

DEAE Sephadex A25 (600 mg) was stirred in 75 ml of 1 M sodium acetate to preserve the acetate ion.

DEAE Sephadex G25.

DEAE Sephadex G25 (600 mg) was stirred in 75 ml of 1 M sodium acetate to preserve the sodium form.

2.5.4.2.3.Preparation of barium acetate/ lead acetate reagen

Barium acetate (2.54 g) and lead acetate (3.78 g) were dissolved separately each in 20 ml of water and then mixed. This reagent was used as a protein, organic acid and sulphate precipitating agent (Thies 1978,1980) since these were otherwise detrimental to sulphatase activity.

2.5.4.2.3. Purification of sulphatase

Sulphatase (aryl-sulphate sulphohydrolase enzyme, Sigma), 1400mg was dissolved in 60 ml of dw. Sixty ml of ethanol was added, centrifuged at 10,000 rpm (8000 g) for 15 min and the precipitate discarded. To

the supernatant, 1.5 volume ethanol was added and the solution was centrifuged at 8000g for 15 min, then the precipitate was dissolved in 40 ml of dw. The solution was passed through mini-columns containing 1 ml (30 mg dry weight) of DEAE-Sephadex A-25 (acetate form) and S-P Sephadex G-25 (sodium form). The eluate was collected, divided into small aliquots and stored at -20°C.

2.5.4.3.Extraction of glucosinolates

Extraction of intact glucosinolates was performed in boiling methanol based on the methods described by Heaney *et al.* (1986). Freeze-dried plant tissue from 20 plants and 0.2 mg of glucotropaeoline[1] (benzyl glucosinolate) as an internal standard were suspended in 20 ml of boiling 80% v/v methanol above boiling water. The internal standard was introduced before homogenization in case of insufficient inactivation of myrosinase. This was heated in a boiling water bath, (taking care it did not overflow), for 10 min to inactivate the endogenous enzyme myrosinase and to extract glucosinolates. After grinding with a pestle and mortar (BDH) the cooled extract was filtered through filter paper (Whatman no 2), on a Buchner funnel under vacuum. The soluble extract was collected in a 250 ml Vacuum flask, a further 20 ml of boiling methanol was added and the residual plant material on the filter paper was re-extracted for 5 min and again filtered. The filtrates from both extractions were combined, the methanol was removed by rotary evaporation in *vacuo* with Rotavapor-R Buch at 40°C, and the residual solution was made up to the final volume of 5 ml with dw.

2.5.4.4. Purification of glucosinolates

Before purification, a cleaning step with diethyl ether was performed. To the 5 ml extract, 1:1 v/v of diethyl ether (BDH) was added and the mixture was gently shaken. This was left for 2 min, then the ether phase (top) was disposed of. This step was repeated twice.

One ml of the cleaned aliquot with 1 ml of water was taken and to this was added 0.1 ml of 0.5M barium and lead acetate solution ml and left for 10 min. The resulting suspension was centrifuged at 3,000 rpm for 5 min and 1 ml of the supernatant was applied to a 100 mg DEAE Sephadex A-25 (acetate form) column (bed volume 1ml) using a Pasteur pipette. Glucosinolates were absorbed onto an ion-exchange (A-25) column and then the column was washed once with approximately 2 ml of dw to remove non-anionic materials and allowed to drain. It was then washed with 2 X 0.5 ml of 0.02 M NaAcetate buffer and drained. A small tube was put underneath the column and finally 75µl of purified aryl-sulphatase solution added to liberate uncharged desulphoglucosinolates (Thies,1979) and left overnight (16-18h). The following day, 3 X 0.5 ml of water was added, draining the column between each addition, and collecting 1.5 ml of the eluate containing the desulphoglucosinolates. The samples were stored at -20 °C until required for analysis.

1Glucotropaeolin does not occur naturally in *Brassica napus*

2.5.4.5. Separation of desulphoglucosinolates by HPLC

Desulphoglucosinolates were separated by High Performance Liquid Chromatography (HPLC) reversed phase mode in which the mobile phase is more polar than the stationary phase (Hamilton & Sewell, 1977). Relatively non-polar compounds elute later than the polar compounds. This was performed using a Waters Associated HPLC system equipped with two chromatography pumps, an injection unit and a gradient controller system. The data were taken using a Water 486 Tuneable Absorbance Detector (set at 230nm) which was connected to a Water Millipore Data Module. The conditions used to separate the desulphoglucosinolates were as described by Heaney *et al.* (1986).

The solvent system was a linear gradient type system (Table-2.6a-) consisting of two solvents(mobile phases):

Solvent A — De-ionised water

Solvent B — 20% acetonitrile (v/v) in solvent A

HPLC column: PHASE SEP (S5 ODS2, 25cm X 46mm)

Table -2.6a-Gradient HPLC table for desulphoglucosinolates

Time (min)	Flow (ml/min)	Solvent A (%)	Solvent B (%)	Gradient
initial	1.5	99	1	-
1	1.5	99	1	None
21	1.5	1	99	Linear
24	1.5	1	99	None
29	1.5	99	1	Linear
39	1.5	99	1	None
39.10	1.5	99	1	-

The solvents were filtered under vacuum through a 0.45μm millipore filter (Type HV) and were degassed with helium.

2.5.4.6.Identification of desulphoglucosinolates

To identify the individual glucosinolates obtained by HPLC analysis their retention times were compared with the retention times of standards (obtained by Dr. J.Rossiter, Wye College). The retention times of seven authentic glucosinolates (progoitrin, sinigrin, gluconapin, glucotropaeolin, glucobrassicin, gluconasturtiin and neoglucobrassicin) were obtained by HPLC analysis using the above parameters. 4-MeOH-glucobrassicin, 4-OH glucobrassicin and Napoleiferin standards were not available and were identified by comparing their retention times of other standards running before them. 4-MeO-glucobrassicin run 30 sec after glucotropaeolin; 4-OH-glucobrassicin run 2 min after gluconapin and napoleiferin 1min before gluconapin). (Sang *et al.*,1984).

2.5.4.7. Quantification of individual glucosinolates

Calculations of individual glucosinolates in each chromatogram were based on internal standards using published response factors for each glucosinolate relative to an internal standard (Lewis & Fenwick,1988). All runs were made at wavelength 230nm, but not all glucosinolates absorb similarly at this wavelength. For this reason, response factors were determined relative to glucosinolate standard (response factor=1) at this wavelength. Aliphatic glucosinolates absorb less and for this reason their response factors are greater than 1 to correct the error; indole glucosinolates absorb more and have response factors lesser than 1. An arbitrary response factor of 1.05 was used for all the unidentified glucosinolates. Table- 2.6b- gives the published response factors for all glucosinolates under this study relative to benzyl glucosinolate.

Table-2.6b-. Published response factor for each individual glucosinolate identified in this study

GLUCOSINOLATE	RESPONSE FACTOR
Progoitin	1.15
Napoleiferin	1.05
Glucotropaeolin	1.00
Gluconasturtiin	1.00
Glucobrassicin	0.31
Neoglucobrassicin	0.21
4-MeOH-glucobrassicin	0.25
4-OH-glucobrassicin	0.29
Unknown peak 1	0.21*
Unknown peak 2	1.05#
Unknown peak 3	1.05#
Unknown peak 4	1.05#
Unknown peak 5	1.05#

*This peak came after Neoglucobrassicin (possibly an indole glucosinolate) and for this reason an arbitrary response factor <1 was used. #Arbitrary response factors. Each chromatographic peak (corresponding to each individual glucosinolate) was compared with the peak of the internal standard with retention time different than the other glucosinolates in the sample and with known quantity. Quantifications were made using the following formula:

(AG/AIS) X RF X µmoles of IS X DF X 1/EX.EF X1/gfdw

AG: Area of each individual glucosinolate
AIS: Area of internal standard
RF: Response factor for each individual glucosinolate relative to internal standard
DF: Dilution factor=Total volume of extract/volume injected to HPLC=(15 X100)/20 =75
EX.EF: Extraction efficiency=Area of internal standard /Area of external standard
gfdw:grms of freeze dried weight

3. Results

3.1. Characterization of the pathogen

3.1.1. Morphological differences between haploid *V.dahliae* isolates and isolates of the diploid *V.d.longisporum* strain

3.1.1.1.Differences in conidial lengths

Seven-day old ungerminated spores were measured under a microscope using an eye-piece micrometer as described in section 2.4.1.3. Two different population groups were observed. All isolates of the first group possessed conidia of 5.5 μm or less (Plate -3.1a-), while those of the second group possessed conidia of 7 μm or more (Plate -3.1b-). The second group included the isolate 195 (*V.d.longisporum*, Stark, 1961) and the two isolates 161 and 162, from Sweden, all of which were proved to be stable diploids by Jackson & Heale (1985). For this reason, the second group was divided into two sub-groups. The sub-group B1 consisted of the three verified diploids (above) and the sub-group B2 containing all those remaining isolates with significantly longer spores. The means for the lengths of spores for the isolates of the three groups are given in Table -3.1-. Application of the t-test to the spore lengths of group A and group B1 showed a very highly significant difference ($P<0.001$). There was also a small significant difference ($P<0.01$) in the length of conidia between the isolates of the sub-group B2 and the group of the three previously verified diploid isolates (sub-group B1) but this is not considered to represent a real distinction between these two groups (see figure -3.1-). Moreover the small no. of replicates in B1 invalidates a standard error calculation for the population of this sub-group. The length of conidia of B2 was highly significantly larger ($P<0.001$) than those of the A group, giving a first indication of diploidy in these isolates and therefore might be considered as putative isolates of the *V.d.longisporum* (Stark) strain (Table-3.2).

Table-3.1-. Mean spore lengths of isolates used in the investigation

GROUP A. Small ('normal')-spored isolates, initially all presumed to be haploid

ISOLATES	HOST	MEAN ± STANDARD ERROR μm
130	Tomato	4 .000±0.093
133	Tomato	5.350±0.073
140	Eggplant	4.585±0.046
362	Shepherd's purse	5.477±0.052
G1	Clover	3.797±0.060
G16	Potato	3.902±0.050
G17	Stock	3.587±0.058
G22	OSR	4.602±0.061
GR1	Olive-tree	4.246±0.094
111	Brussels sprout	4.620±0.130

GROUP B1. Large-spored isolates, previously verified as diploid *V.d.longisporum* (Jackson and Heale, 1985).

ISOLATE	HOST	MEAN ± STANDARD ERROR µm
195	Horse radish	8.971±0.197
161	OSR[1]	8.505±0.145
162	Sugar beet	8.400±0.088

[1]oilseed rape

GROUP B2. Large-spored isolates, possible diploids by comparison with *V.d.longisporum.* (see discussion)

ISOLATE	HOST	MEAN ± STANDARD ERROR µm
86207*	Chinese radish	8.470±0.156
84013*	Chinese cabbage	7.997±0.070
84120*	Chinese cabbage	8.312±0.151
G10	OSR	7.910±0.198
G18	OSR	7.542±0.061
G19	OSR	9.100±0.114
G25	OSR	7.945±0.100
G23	OSR	8.557±0.149
G29	OSR	7.282±0.136
G34	OSR	7.997±0.220
GV165	OSR	7.194±0.136
GV166	OSR	7.656±0.146
GV167	OSR	7.788±0.108
GV168	OSR	7.788±0.136
GV169	OSR	7.612±0.111
GV170	OSR	7.678±0.110
F1	OSR	7.210±0.524
G334	OSR	7.926±0.131
F617	OSR	7.326±0.102
F617-7	OSR	7.968±0.147
F617-9	OSR	7.612±0.086
P671	OSR	7.616±0.087
F654-2	OSR	8.144±0.105
G856	OSR	7.480±0.089
GV	OSR	8.830±0.220
G859	OSR	8.078±0.121

* Reported by Horiuchi *et al.* (1990) as being *V.d.longisporum* although diploidy was not verified by them.

Table-3.2-. T-test data for spore lengths for the groups in table-3.1-

GROUP	SAMPLE SIZE	MEAN µm	GROUP	t-VALUE	P-VALUE
A	10	4.417	A-B1	10.843	p<0.001
B1	3	8.625	B2-B1	2.596	p<0.01
B2	26	7.885	A-B2	17.772	p<0.001

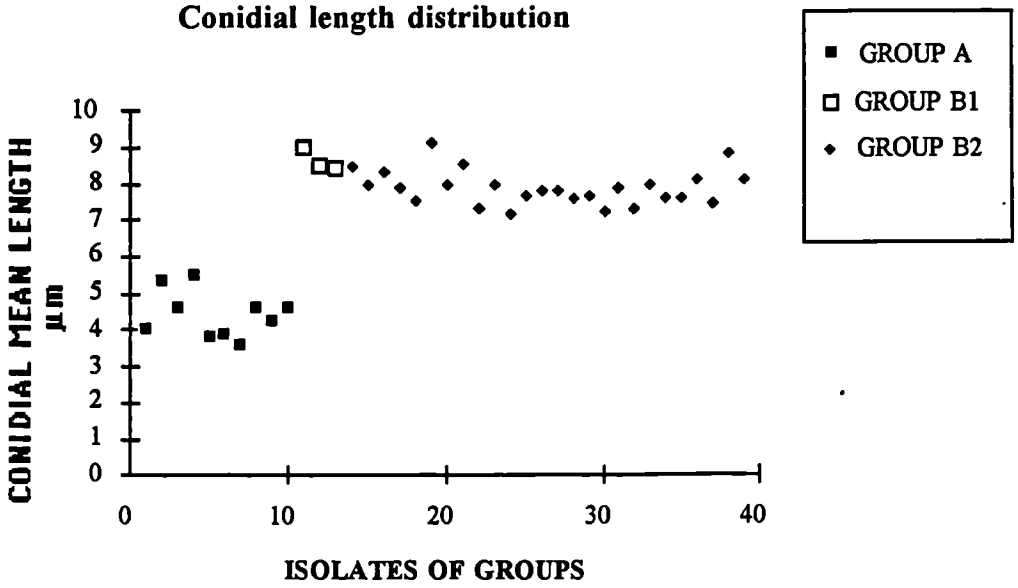


Figure-3.1- .Conidial length distribution of isolates of three groups, A, B1, B2 under test.

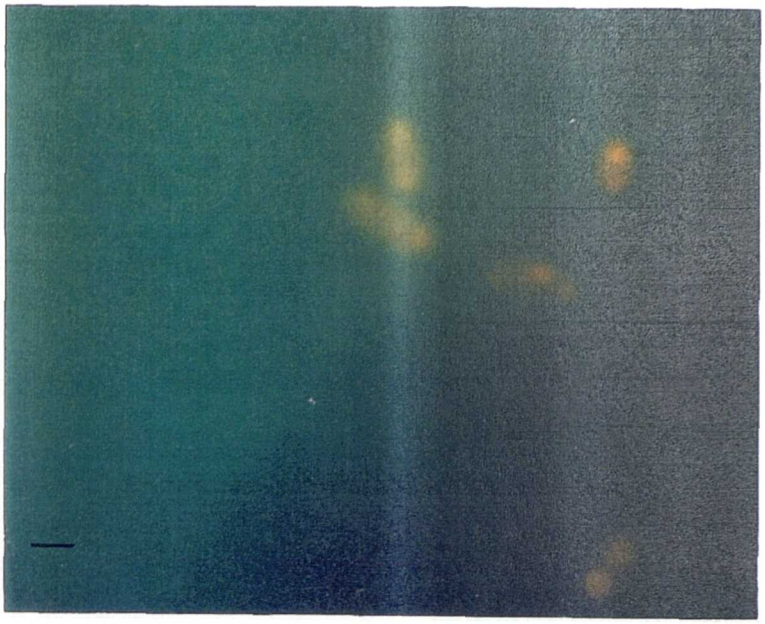


Plate -3.1a-. Conidia from an haploid isolate of *V.dahliae* (130), group A, with mean spore length 4.000 ± 0.093 µm. Conidia were treated with FDA and observed under fluorescent light microscopy (bar=1.5µm).

Plate -3.1b-. Conidia from a diploid isolate viz *V.d.longisporum* (161), group B1, with mean length 8.505 ± 0.145 µm. Conidia were treated with FDA and observed under fluorescent light microscopy (bar = 2µm).

3.1.1.2. Differences in dark resting bodies (microsclerotia)

The morphological differences appertaining to microsclerotia were studied on three different media as described in 2.4.1.1. The formation and the development of these structures was first investigated on 10 day-old PDA cultures. On PDA medium all isolates in table-2.1b, except the two isolates of *V. albo-atrum* (which formed dark resting mycelium) began to form black microsclerotia in 4-5 days. During the formation of microsclerotia, 3 different stages of development were distinguished in both types of *V.dahliae* (Table-3.3-).

Stage 1.

Initiation. (Hall & Ly, 1972). In this stage, individual hyphae started to become more regularly septate and became swollen forming hyphae of torulose cells. This was observed at the margin of the fungal colony on PDA medium (0.25 cm from the hyphal tips). Isolates of group A became less regularly septate than isolates of groups B1 and B2. Plates -3.2a-, -3.2b- show microsclerotial initiation in isolates of haploid and diploid strains respectively in stage 1.

Stage 2.

Formation. Single or contiguous swollen hyphae formed a structure of almost spherical cells by repeating budding (isolates of group A) and/or aggregation of several hyphae (isolates of groups B1 and B2). This was observed 0.5 cm from the hyphal tips. Plates -3.3a-, 3.3b-, show microsclerotia formation in isolates of haploid and diploid strains respectively in stage 2.

Stage 3.

Maturation. Structures became dark by deposition of an allomelanin pigment within the walls of the microsclerotial cells. This was observed 0.5 cm from the hyphal tips. Plates -3.4a-, -3.4b- show microsclerotial formation in isolates of haploid and diploid strains respectively in stage 3.

Due to the differentiation at stage 2 of the microsclerotial development two different major structural types were finally formed.

TYPE M1

Type **M1** microsclerotia were formed by isolates of the diploid strain viz. *V.d.longisporum.* The budding at stage 2 was mainly two dimensional and hyphae aggregated forming a strand-like elongate structure of distinct rounded cells. These strand-like structures at later developmental stages were lined-up and connected with threads of darkly pigmented swollen cells, forming a 'web' of microsclerotia embedded in the medium, that could be easily observed under a dissecting-stereo microscope. These strand-like elongate structures were developed in radiating lines in the PDA medium giving to the culture a 'knobbly fibrous' appearance. Some darkened hyphal cells were often observed in immediate contact with such microsclerotia (Plate 3.6d).

TYPE M2

Type **M2** microsclerotia were formed by isolates of haploid strains of *V.dahliae.* The budding at stage 2 was three-dimensional, forming an organized compact mass of spherical cells, being an almost globose structure. These more or less globose structures were developed individually later and were

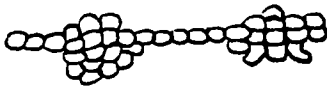
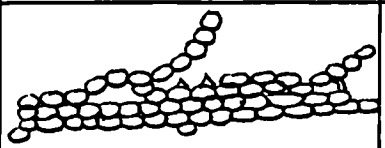
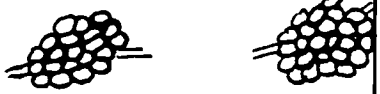
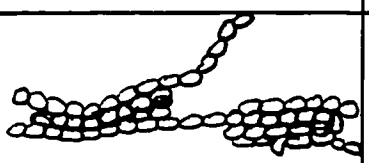
scattered on the PDA medium giving to the culture a 'gritty' appearance, a term first coined by Talboys,(1960). Thus, strands of darkly pigmented swollen hyphae linking mature microsclerotia were not observed here, see Plate -3.6c-.

Isolate 235 of *V.albo atrum* formed dark resting mycelium 2.5 cm in from the hyphal tips, 8 days after inoculation. There was no fungal differentiation at the margin of the hyphal colony and no budding or aggregation was observed as in type **M1** and type **M2** microsclerotia. Resting mycelium formed from hyphae that became regularly septate and darkened by pigmentation, giving to the culture a 'fibrous' appearance (Talboys 1960). It was very difficult to observe the resting mycelium from undisturbed cultures under low magnification power (X125) and for this observation a sample was taken from the dark area of the colony and examined under higher magnification, see Plate -3.5d-.

Plates -3.6a- and -3.6b- show mature microsclerotia on rape medium.

Plates -3.6c- and -3.6d- show mature microsclerotia on PDA medium.

Table 3.3- Schematic representation of microsclerotia formation of isolates of the three groups at the three stages of development.

GROUP A	GROUPS B1,B2	STAGE
		1. INITIATION
		2. FORMATION
		3.MATURATION

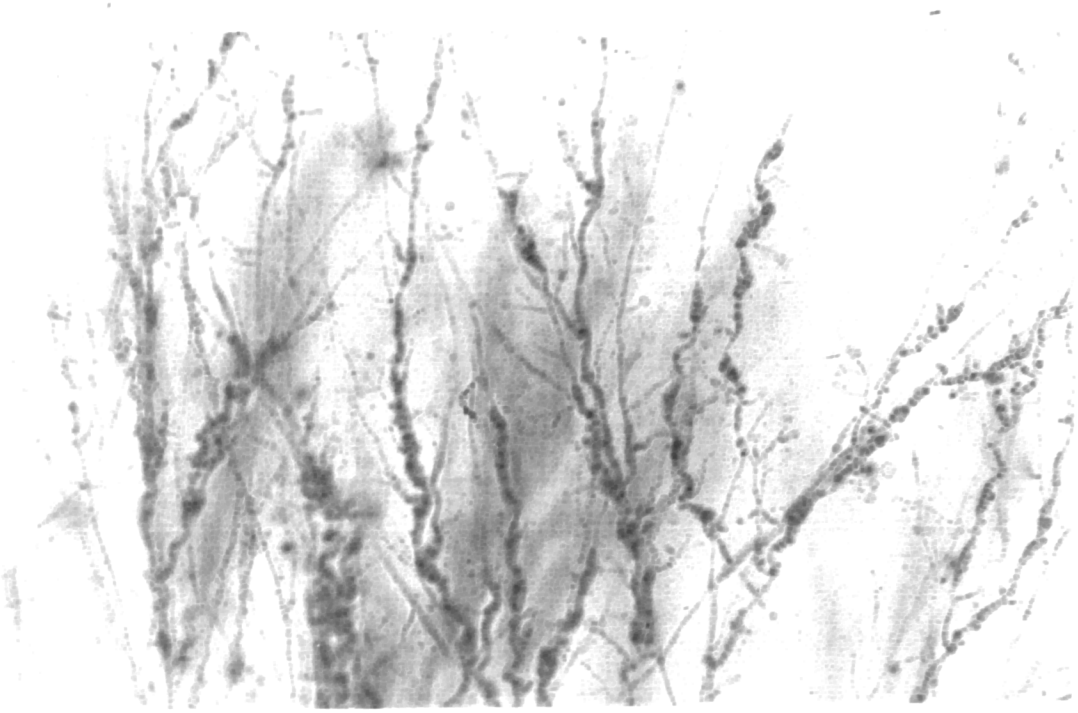


Plate-3.2a-. Stage 1 (initiation) of microsclerotial formation in an isolate (G17, stock) of group A shown later to be an isolate of the haploid strain of *V.dahliae*. Hyphae close to the hyphal tips of a PDA colony (0.25 cm from the margin) started to become septate and swollen (magnification factor X 125).

Plate-3.2b-. Stage 1 (initiation) of microsclerotial formation in an isolate (161, sugar beet) of the diploid strain viz. *V. d. longisporum* (X 125).(Same as Plate -3.2a- haploid at stage 1).

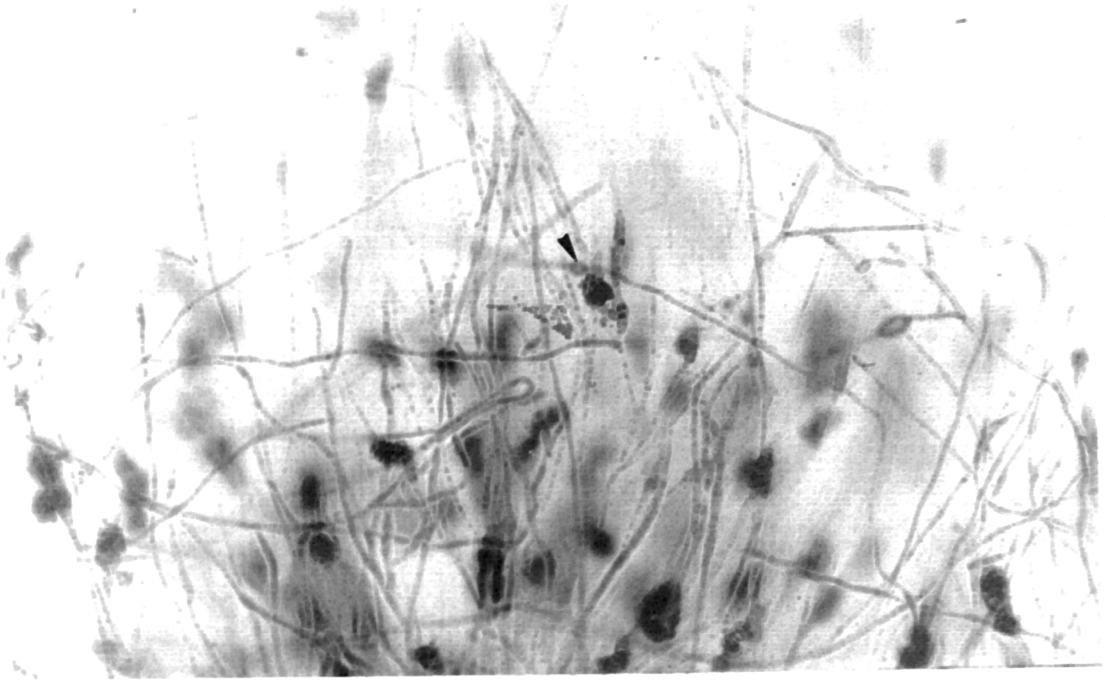


Plate-3.3a-. Stage 2 of microsclerotial formation in an isolate (130, tomato) of *V.dahliae* (haploid, group A). Swollen cells start forming a globose spherical structure (arrow-head) by repeated budding (X125).

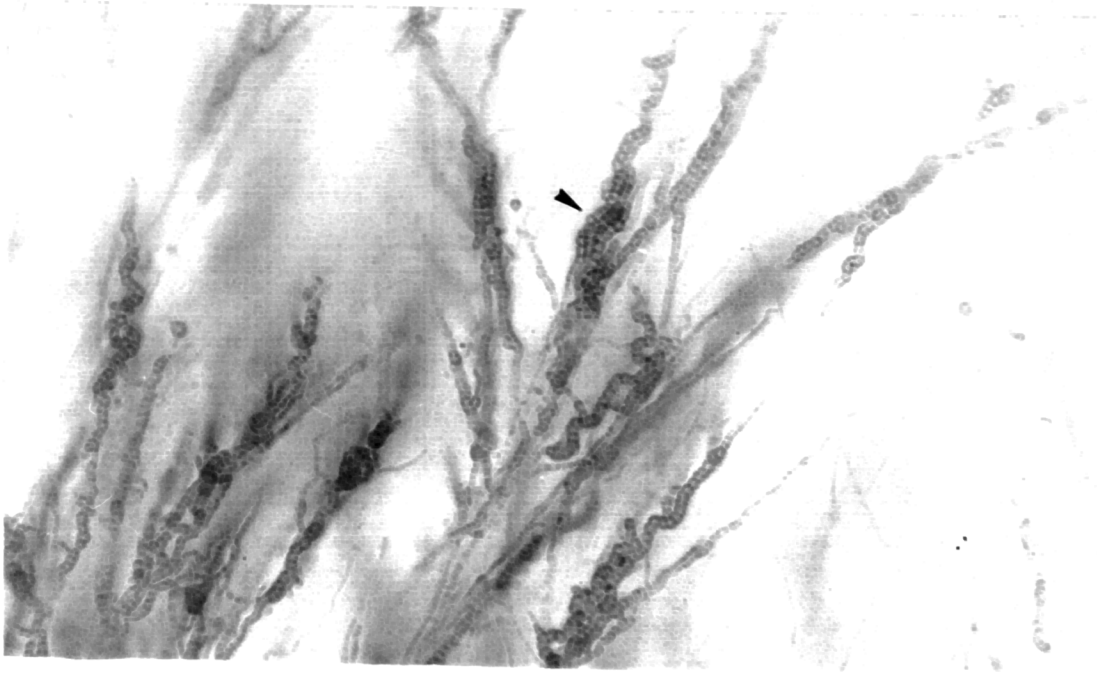


Plate -3.3b-. Stage 2 of microsclerotial formation in an isolate (GV170, OSR) of group B2 shown later to be an isolate belonging to a diploid strain viz. *V.d.longisporum*. Hyphae aggregated (arrow-head) and forming elongate strand-like structures (X125).

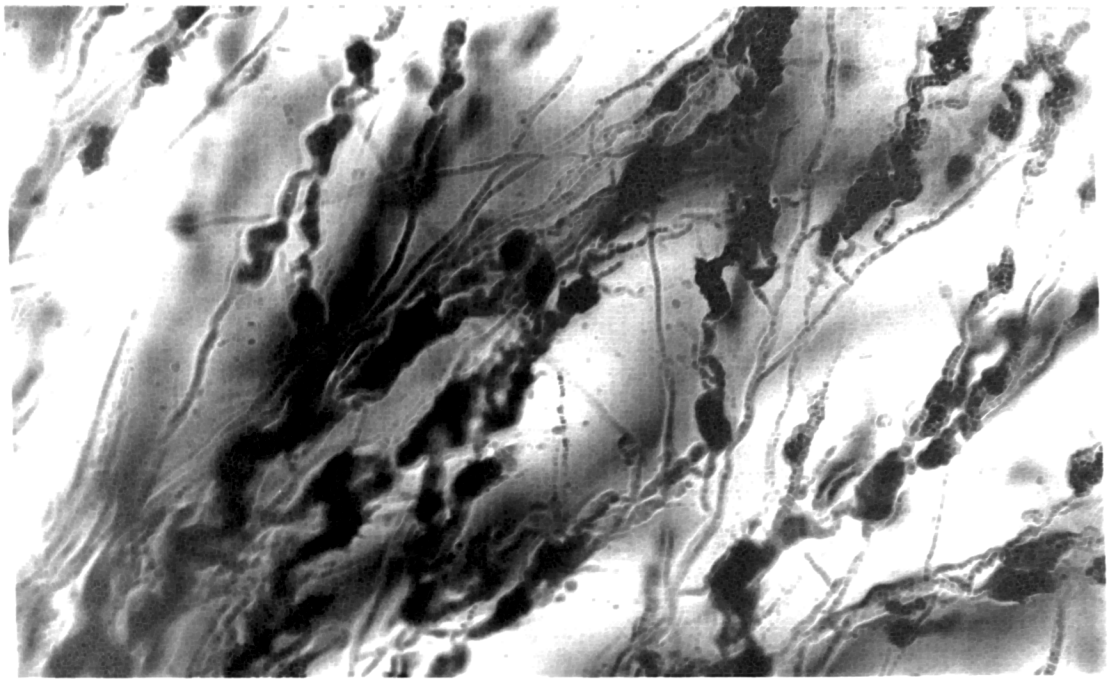


Plate-3.4a-. Early stage 3 of microsclerotial formation in an isolate (G17, stock) of group A shown later to be an isolate belonging to an haploid strain of *V.dahliae*. Microsclerotia are of type M2, becoming more or less globose in shape (X125).

Plate -3.4b-. Early stage 3 of microsclerotial formation in an isolate (GV170, OSR) of group B2 shown later to be an isolate belonging to a diploid strain viz. *V.d.longisporum*. Microsclerotia are of type M1 remaining as strand-like, elongate structures with considerable aggregations forming laterally between adjacent hyphal strands (X125).

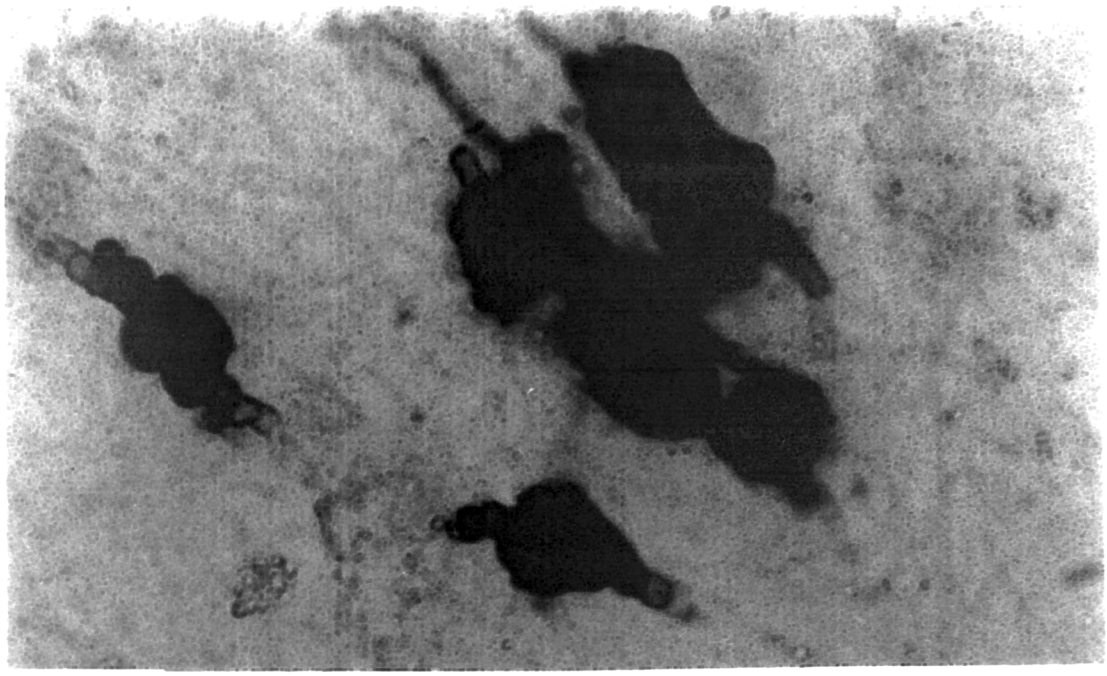


Plate-3.5a-. Mature, 3-week-old microsclerotia, type **M2** developed on PDA medium (isolate G1, haploid, group A, X625).

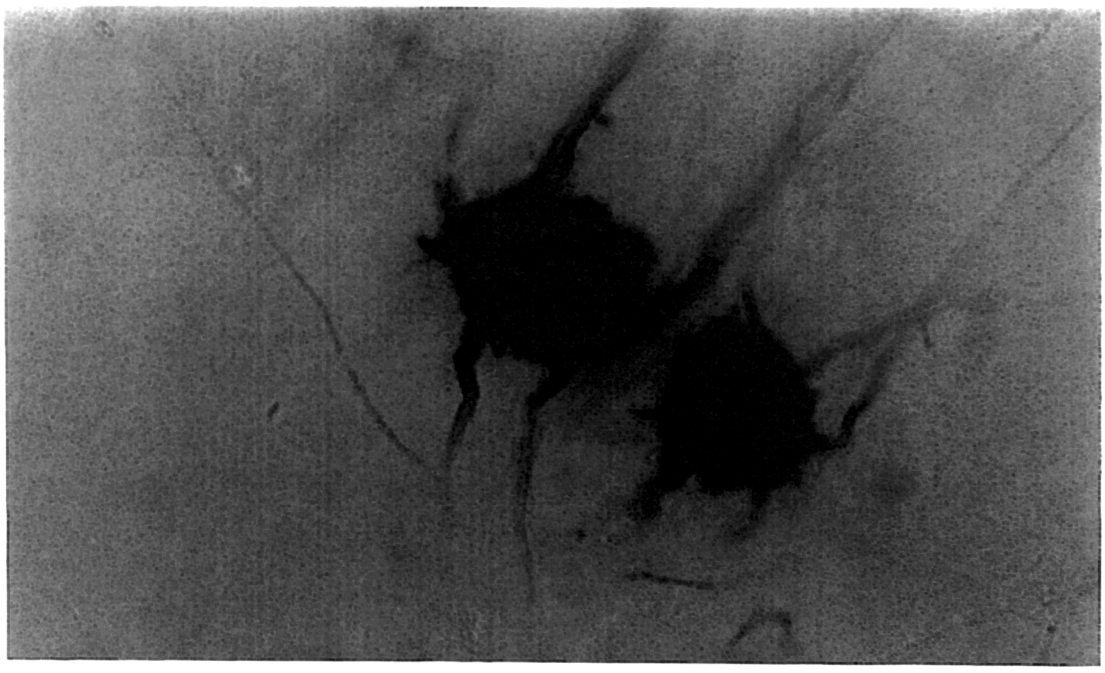


Plate -3.5b-. Mature, 3-week-old microsclerotia, type **M2** developed on PDA medium (isolate G19*, group B2), (X312.5).

* An intermediate strain ,shown later to be a putative recombinant of *V.d. longisporum.*

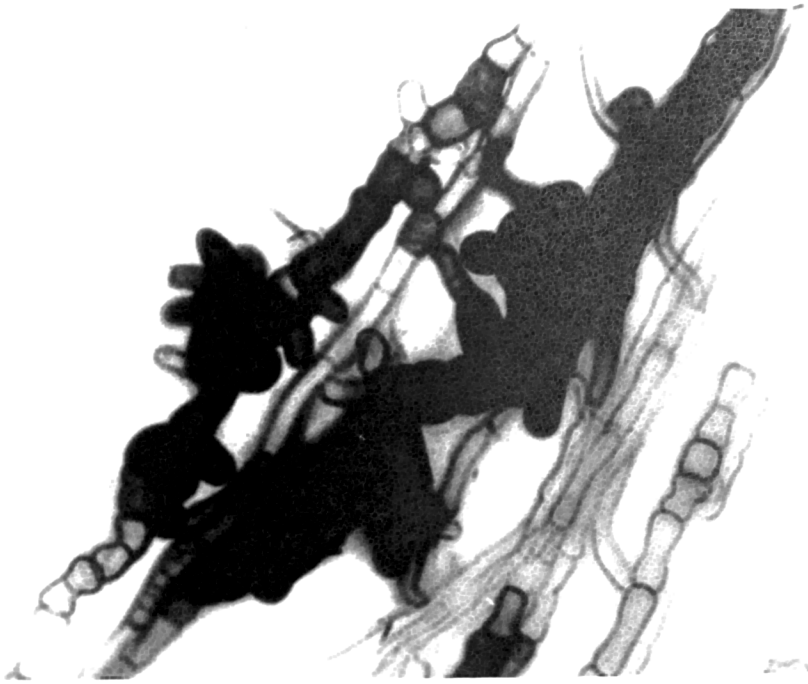


Plate -3.5c-. Mature, 3-week-old microsclerotia of type **M1** developed on PDA medium (isolate F1, diploid, group B2, X625).

Plate -3.5d-. Black resting mycelium (3 weeks after inoculation) developed on PDA medium (isolate 235 of *V.albo-atrum*, X 625).

Microsclerotia were observed on four different media viz. : Hall and Ly, PDA, Rape and Prune lactose media. After inoculation for 20 days in the dark, the two types of fully mature microsclerotial forms were clearly distinguishable under a stereo-dissecting microscope. Plates -3.6a- and -3.6b- show microsclerotia formation in an isolate (130, haploid) of *V. dahliae* and an isolate (161) of the diploid strain viz. *V. d. longisporum* respectively on rape medium.

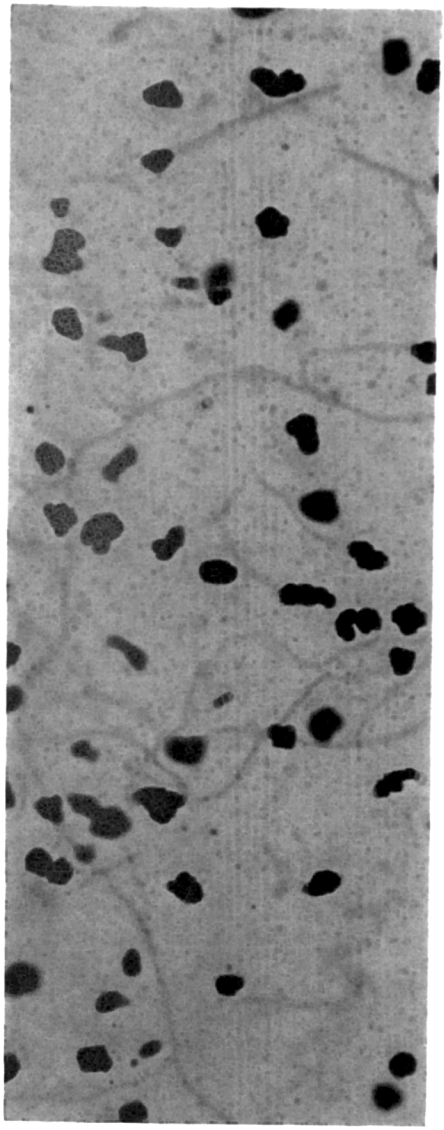


Plate-3.6a-. **Plate -3.6b-.**

Plate-3.6a-.Mature microsclerotia on rape medium of an isolate (130, tomato) of *V.dahliae* (haploid). Compact, more or less globose, mature microsclerotia of type M2, are seen (X125)

Plate-3.6b-.Mature microsclerotia on rape medium of an isolate (161, sugarbeet) of the diploid strain viz. *V.d.longisporum*. Irregular, elongate (much less compact) microsclerotia of type M1, are seen (X125).

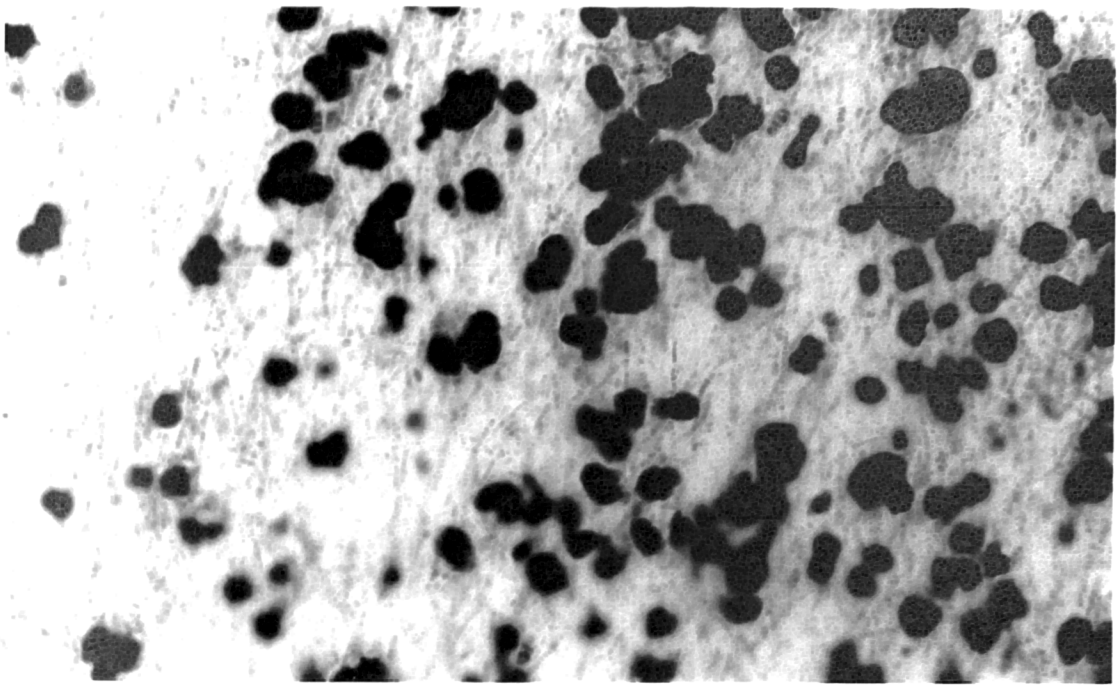


Plate-3.6c-. Fully mature, 3-month-old microsclerotia on PDA medium of an isolate (G22, OSR) of group A later shown to be an isolate of the haploid strain *V.dahliae*. Globose compact microsclerotia of type **B**, individually formed, are seen (X312.5).

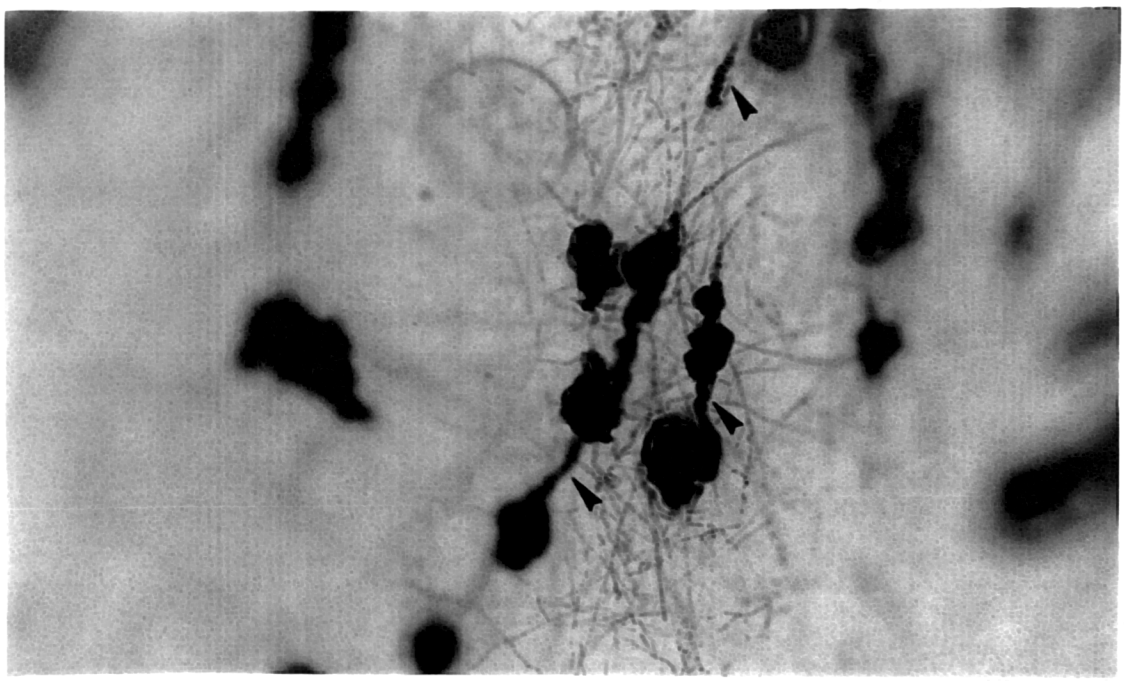


Plate -3.6d-. Fully mature, 3-month-old microsclerotia on PDA medium of an isolate (G334, OSR) of group B2 later shown to be an isolate of the diploid strain viz. *V.d.longisporum*. Microsclerotia of type **M1**, connected with black swollen cells and black hyphae (not seen in plate -3.6c- above), are seen (X312.5).

Table-3.4- Type of microsclerotia[1] as formed on 4 different media

GROUP A	ISOLATE	HALL & LY	PDA	PYA	RA
V.dahliae	130	Type M2	Type M2	Type M2	Type M2
V.dahliae	133	NP[2]	Type M2	Type M2	NP
V.dahliae	140	NP	Type M2	Type M2	Type M2
V.dahliae	362	Type M2	Type M2	Type M2	NP
V.dahliae	G1	Type M2	Type M2	Type M2	Type M2
V.dahliae	G16	NP	Type M2	Type M2	NP
V.dahliae	G17	NP	Type M2	Type M2	NP
V.dahliae	G22[a]	NP	Type M2	Type M2	NP
V.dahliae	GR1	Type M2	Type M2	Type M2	Type M2
V.dahliae	111[b]	NP	Type M2	Type M1?	NP
GROUP B1	**ISOLATE**	**HALL & LY**	**PDA**	**PYA**	**RA**
V.d.longisporum	195[c]	NP	Type M2	NP	NP
V.d.longisporum	161	Type M1	Type M1	Type M1	Type M1
V.d.longisporum	162	No Growth	Type M1	NP	Type M1
GROUP B2	**ISOLATE**	**HALL & LY**	**PDA**	**PYA**	**RA**
V.d.longisporum ?	86207[d]	Type M1	Type M1	Type M1	Type M1
V.d.longisporum ?	84013[d]	Type M1	Type M1	Type M1	Type M1
V.d. longisporum ?	84120[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G10[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G18[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G19[e]	NP	Type M2 ?	Type M2 ?	Type M2 ?
V.dahliae	G23[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G25[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G29[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G34[d]	Type M1	Type M1	NP	Type M1
V.dahliae	GV165[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	GV166[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	GV167[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	GV168[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	GV169[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	GV170[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	F1[d]	Type M1	Type M1	Type M1	NP
V.dahliae	G334[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G856[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	F617-7[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	F617-9[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	F654-2[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	G859[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	P671[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	GV[d]	Type M1	Type M1	Type M1	Type M1
V.dahliae	F617[d]	Type M1	Type M1	Type M1	Type M1

[1] For descriptions of type A and B see Table-3.1-.
[2] Not produced.
[a] Although diploid when first isolated, became haploid after two years.
[b] Probably still diploid, but more or less sterile and showing some characters associated with haploidy.
[c] Probably still diploid, but more or less sterile.
[d] Diploid showing characters associated with diploidy.
[e] Haploid, but showing recombinant characters e.g. long spores.

3.1.1.3. Differences in the morphology of conidiophores

Verticillium species form erect conidiophores, bearing phialides in verticils. Conidiophore observations were made from undisturbed cultures as described in section 2.4.1.2.

All isolates tested formed verticillate conidiophores. Conidiophores formed from globose, swollen, initial cells (cells that later formed microsclerotia, in all *V.dahliae* isolates) 0.5 cm away from the edge of the colony (Plate -3.7-). Completely white, hyaline colonies (hyaline variants) were +/- sterile, giving a white cottony appearance to the culture. Isolates of haploid strains of *V.dahliae* often produced hyaline cultures after repeated sub-culture but their conidial production could be maintained by careful selection (single sporing). Isolates of the diploid strain viz. *V.d.longisporum* rarely appeared sterile and consistently produced a large number of conidia.

Isolates of haploid strains of *V.dahliae* usually formed conidiophores with 4-5 verticillate phialides/ node. Five phialides were usually observed at the apex of the conidiophore. Isolates of the diploid strain formed mostly 3 phialides/node, occasionally four; 4 were regularly observed at the apex of the conidiophore. By comparison, *Verticillium albo-atrum*, isolate 235, formed conidiophores from a normal hypha but dark pigment (allomelanin) later formed at the base of the conidiophore being deposited in 3-4 basal cells. Three to four phiallides were formed at each node. At the edge of the *V.albo-atrum* colony, there were no budding cells and the dark resting mycelium developed away from the colony edge (2.5 cm from the hyphal tips). The number of phialides / node of conidiophores for all isolates is given in table-3.5-.

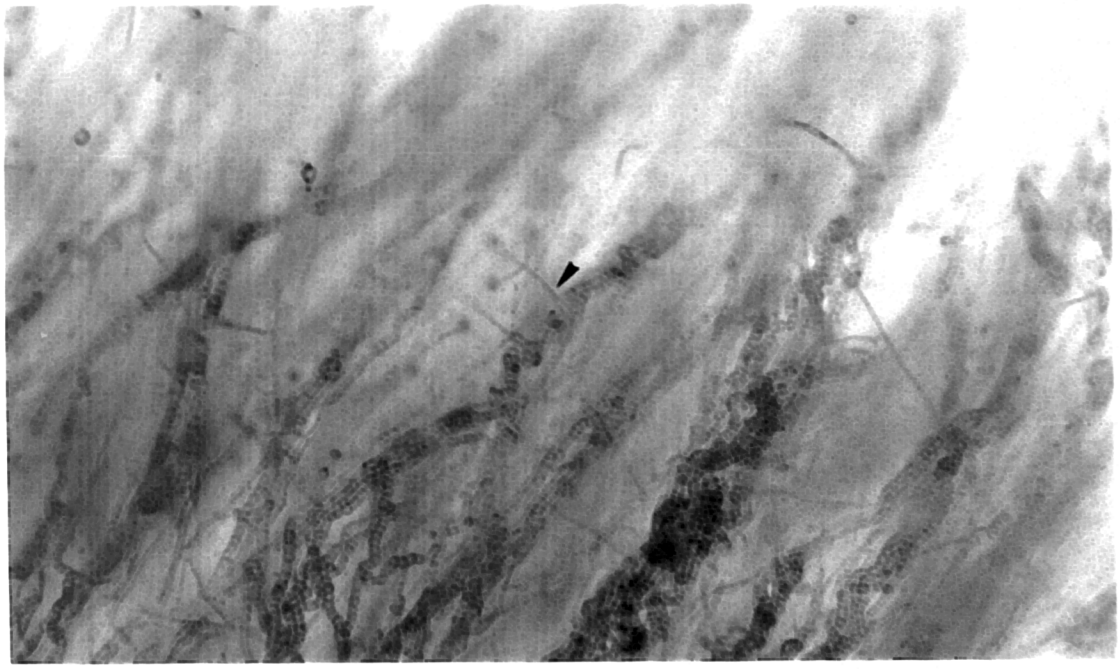


Plate-3.7- Conidiophore formation in a putative diploid isolate (G856) under investigation. Conidiophores were produced from swollen globose cells that finally formed microsclerotia M1, (X125) by a process of lateral aggregation of budding hyphal strands and deposition of dark pigment (allomelanin).

Table-3.5- Number of phialides produced at each conidiophore node.

ISOLATE	PHIALIDES	ISOLATE	PHIALIDES	ISOLATE	PHIALIDES
GROUP A		GROUP B1		GROUP B2	
130	4-5	195[c]	sterile	G18	3
133	4-5	161	3-4	G19[e]	3
140	4-5	162	3-4	G23	3
G22[a]	4-5	GROUP B2		G25	3
362	4-5	84120[d]	3	G29	3
GR1	4-5	84013	3	G34	3
111[b]	sterile	86207	3	GV165	3-4
G1	4-5	F1	3	GV166	3-4
G16	4-5	G334	3	GV167	3-4
G17	4-5	G856	3	GV168	3-4
		F617-9	3-4	*V. albo-atrum*	
		F654-2	3-4	235	3-4
		P671	3		
		F617	3-4		
		G859	3		
		GV170	3		

Groups A, B1 and B2 tentatively divided according to spore length, see Table-3.1-.

[c] See foot note to table -3.4-.
[e] Seefotnote to Table-3.4-.
[a] See footnote to Table -3.4-.
[d] See footnote to Table-3.4-.
[b] See footnote to Table -3.4-.

3.1.2. Ploidy differences in nuclei of various isolates

3.1.2.1. Differences in nuclear diameters of ungerminated conidia

Two different fluorochromes were used for nuclear staining: acridine orange and DAPI . Acridine orange fluorescent staining permits unselective staining of all nuclei (Bainbridge & Roper, 1966). This staining proved to be inferior to DAPI and for this reason DAPI was used for nuclear staining and nuclear diameter measurements as described in section 2.4.2.1.2. DAPI is a specific probe for DNA and does not fluoresce unless bound to DNA (Butt *et al.*, 1989). Conidia of all isolates of *V.dahliae* strains and *V.d.longisporum* examined were shown to be regularly uninucleate (Plate 3.8a, 3.8b).

The results for the three groups are given in table-3.6- and are based on an average of 50 readings per isolate. T-tests showed highly significant differences between the means of the B1 and A groups, as well as between the B2 and A groups. There was no significant difference between the sub-group B1 of isolates, already verified as the diploid strain of *V.d.longisporum* and the putative diploids of the sub-group B2 (table -3.7-).

Table -3.6- Mean nuclear diameter of isolates under test.

ISOLATE	DIAMETER ± S.E. µm	ISOLATE	DIAMETER ± S.E. µm	ISOLATE	DIAMETER ± S.E. µm
GROUP A*		GROUP B1*		GROUP B2*	
130	0.986 ± 0.034	195	1.700 ±0.060	86207	1.940 ±0.040
133	0.990 ± 0.040	161	1.816 ±0.024	84013	1.900 ±0.044
140	1.600 ± 0.050	162	1.800 ±0.040	84120	1.590 ±0.036
362	1.400 ± 0.060			G10	1.790 ±0.033
G1	1.460 ± 0.060			G18	2.101 ±0.030
G16	0.918 ±0.034			G19	1.742 ±0.044
G17	0.918 ±0.017			G25	1.991 ±0.041
G22	1.036 ± 0.037			G23	2.024 ±0.037
GR1	0.997 ± 0.019			G29	1.890 ±0.038
111	no data (sterile)			G34	1.810 ±0.047
				GV165	1.955 ±0.036
				GV166	1.920 ±0.030
				GV167	1.870 ±0.036
				GV168	2.010 ±0.030
		GROUP B2*		GV169	1.830 ±0.040
		F654-2	1.760 ±0.063	GV170	1.701 ±0.039
		G856	1.716 ±0.064	F1	1.900 ±0.039
		G859	1.892 ± 0.050	G334	1.760 ±0.063
		GV	1.830 ±0.039	F617	1.530±0.057
		P671	1.850 ±0.045	F617-7	1.848 ±0.068
				F617-9	1.860 ±0.058

* Groups A, B1, and B2 tentatively divided according to spore-length.

Table-3.7-· T-tests applied to nuclear diameter data for paired groups of all isolates examined.

GROUP	MEAN µm	GROUP	t	P-value
A	1.145	B1-A	3.955	p<0.01
B1	1.772	B2-A	10.565	p<0.001
B2	1.846	B1-B2	0.976	p>0.01

There was highly significant positive correlation between the conidial length and the nuclear diameter of the isolates of the two main groups (see figure-3.2-).

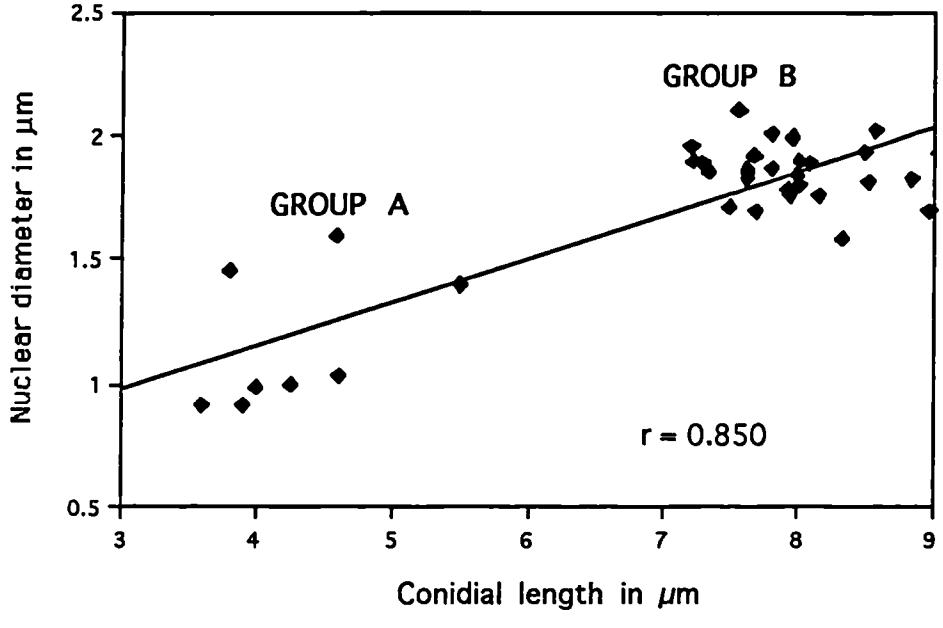


Figure -3.2- Correlation between conidial length and nuclear diameter of isolates of the two main groups (A, B)

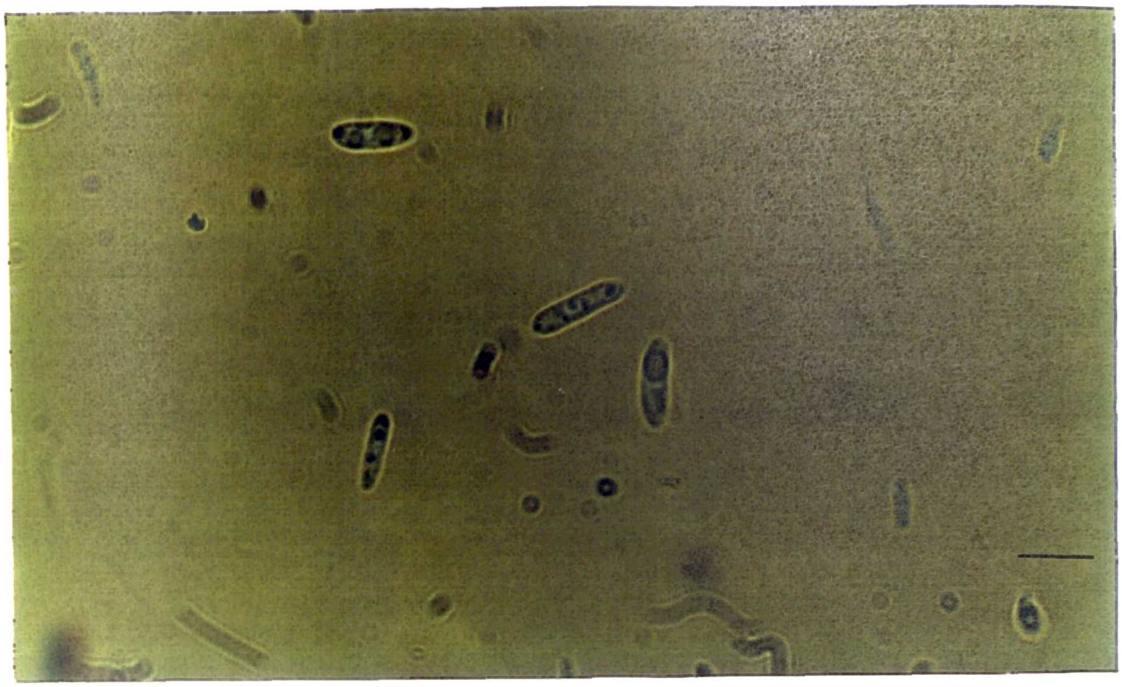


Plate-3.8a- Conidia of isolate (195) of the diploid strain viz. *V.d.longisporum*, group B1, stained with DAPI and visualized using light microscopy (bar=6µm).

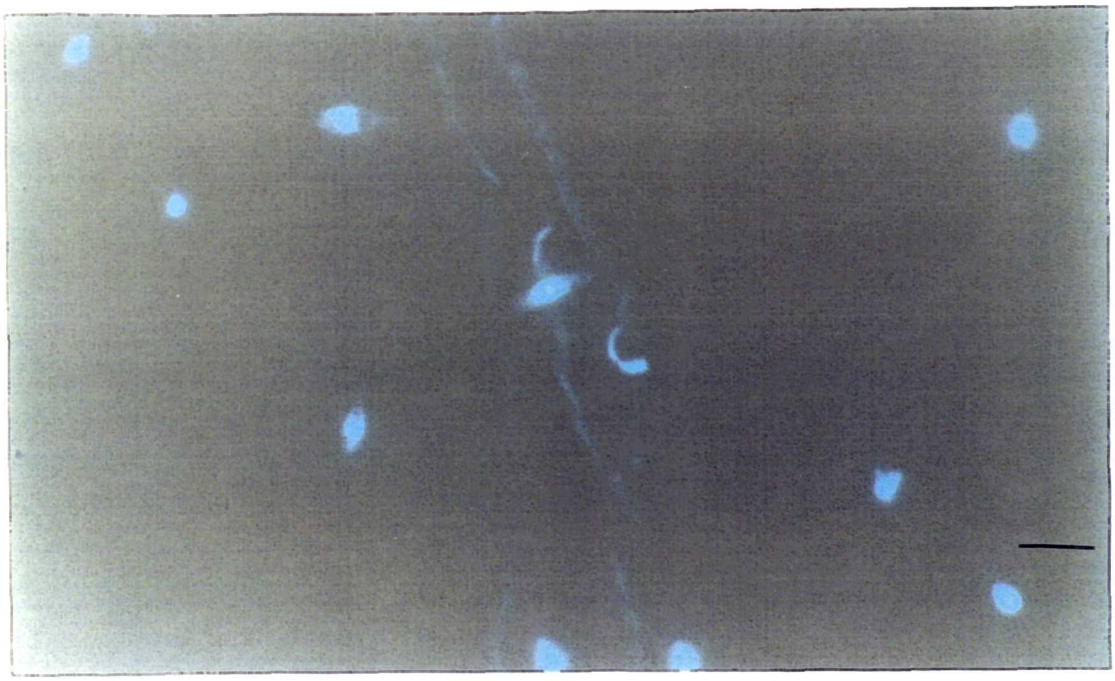


Plate-3.8b- The same conidia as in Plate -3.8a-, stained with DAPI, but visualized with fluorescent microscopy. Each ungerminated conidium contained one nucleus (bar=6µm).

3.1.2.2. Differences in nuclear content between haploid/diploid strains -

To estimate the ploidy levels of conidia, the Feulgen stain procedure was performed (using ungerminated conidia) thus giving a relative measure of the quantity of DNA. The staining method of Jackson & Heale (1985) was found to be generally satisfactory, but was performed with some modifications as described in section 2.4.2.1.3. The steps that were found to be crucial for the results to be consistent were :

-Nuclei of conidia had to be in G1 phase, the period before DNA synthesis. This was obtained by using freshly harvested, ungerminated conidia from 7-day-old, spread (partially synchronized), PDA cultures. Figures 3.5a, 3.5b, 3.5c show DNA stain values in arbitrary units for a large population of conidia (100 spores) for representative isolates of the three groups.

-Cold hydrolysis was performed with 5N HCl at 25^0C for 40 min as indicated by the calibration curve of hydrolysis time against amount of Feulgen/conidiospore nucleus recorded on the scanning microdensitometer (Figure -3.3-).

-All steps, except dehydration at the end, were performed in Eppendorf tubes (not on slides) and this produced a simplified and highly reliable method.

-Washing with SO_2 water after staining was necessary to remove any unbound stain but only for 5 min, otherwise nuclei started to destain giving very low density values and considerable variability.

-An internal standard (isolate 161 of *V.d.longisporum*), which was always incorporated on the same slide, was used for each staining batch in order to avoid staining variation between batches and slides (Figure -3.4-). This permitted a consistent comparison between the isolate under investigation and the isolate 161 of the diploid strain: *V.d.longisporum* under exactly the same conditions.

Feulgen stain revealed uninucleate conidia with spherical, uniformly magenta-stained, nuclei. Measurements were made as described in section 2.4.2.2.3. with a Vickers microdensitometer and the results in arbitrary units are given in Table -3.8-. Conidiospore length measurements of isolates under test placed them in two groups : Group A consisting of isolates with relatively small conidia of 5.5 μm or less, and Group B (B1-B2) of isolates which possessed conidia of 7 μm or more. Feulgen DNA microdensitometry indicated that all isolates of group A were of the same ploidy (haploid) and that isolates of group B were all diploid, except one isolate, G19, that appeared to be haploid. Thus by this procedure all the isolates of group B had nuclei almost with double the amount of DNA, as compared with the amount of conidial DNA of haploid isolates (see Figures 3.6a, 3.6b, 3.6c, 3.6d).

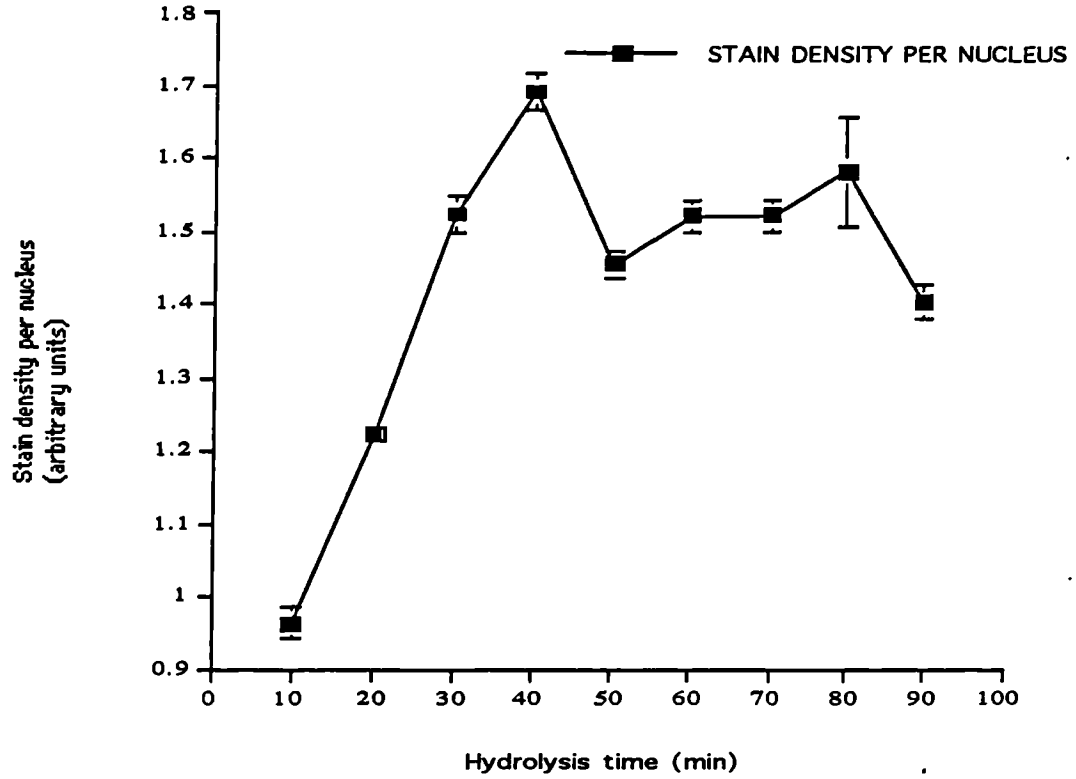


Figure-3.3-. Calibration curve of hydrolysis time against amount of Feulgen stain per conidial nucleus (isolate 161*, diploid: *V.d.longisporum*) recorded on the scanning microdensitometer.

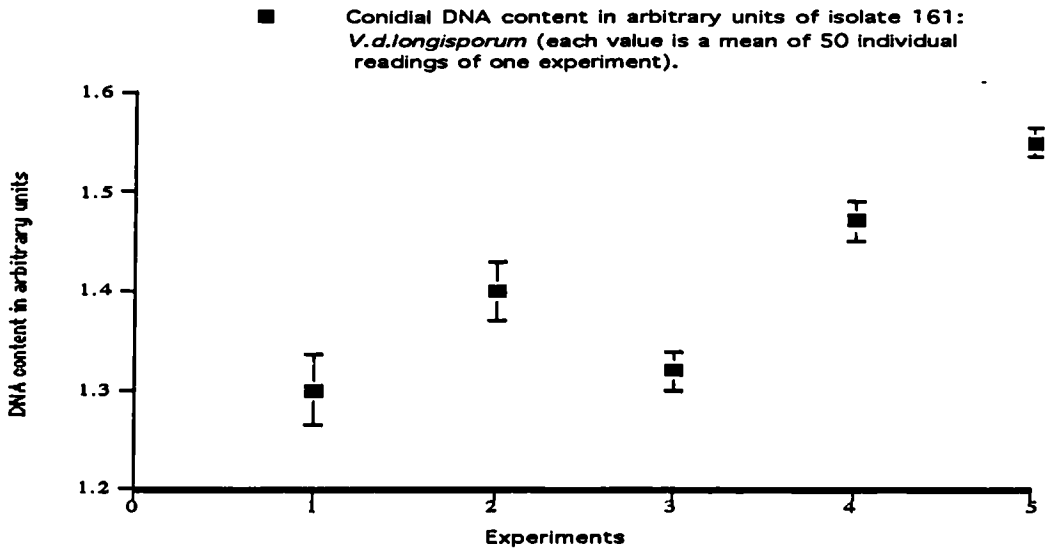


Figure-3.4-. Variable DNA values for conidial nuclei of isolate 161 of the diploid strain viz. *V.d longisporum* from 5 different experiments, indicating the lack of an internal standard.

* This verified diploid strain was used as an internal standard reference when comparing each isolate

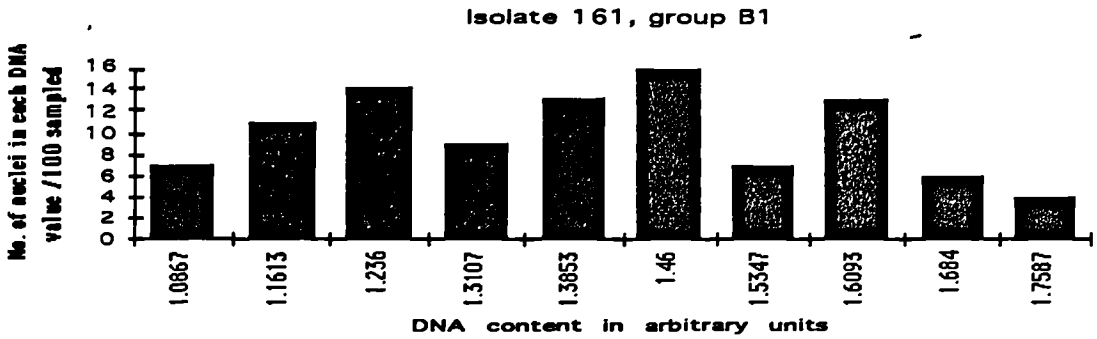


Figure-3.5a-. DNA content/nucleus of 100 individual ungerminated conidia of the isolate 161 of the diploid strain viz. *V.d.longisporum*. Mean value: 1.432 and standard deviation ±0.190.

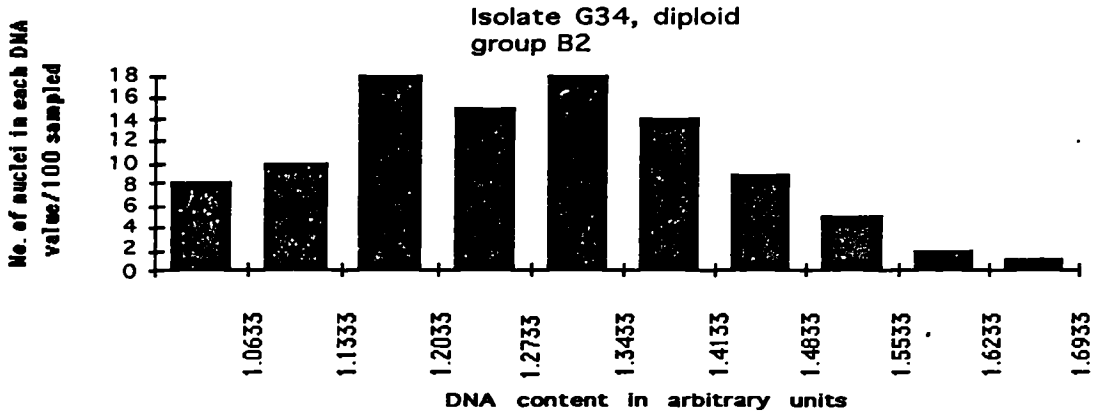


Figure-3.5b-. DNA content /nucleus of 100 ungerminated conidia of isolate G34 of group B2*. Mean DNA value: 1.340 and standard deviation: ±0.150

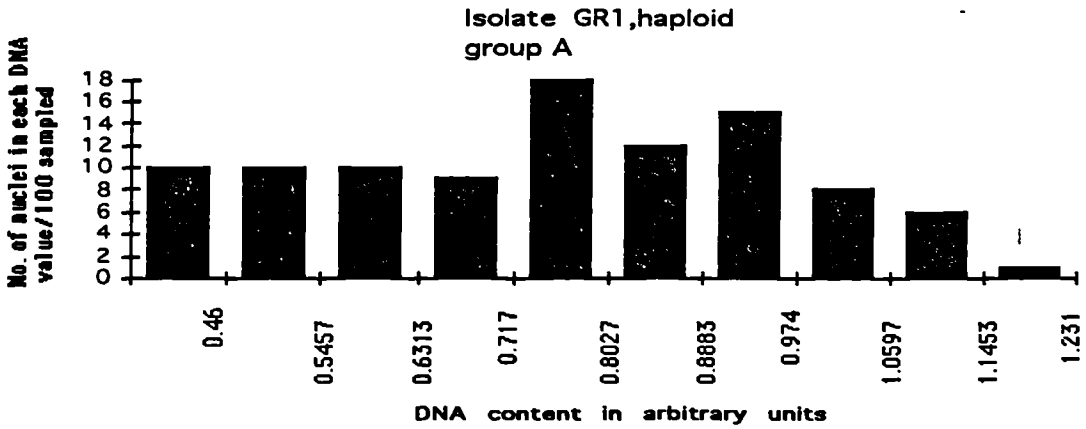


Figure-3.5c-. DNA content /nucleus of 100 ungerminated conidia of isolate GR1 of group A*, an haploid strain of *V.dahliae*. Mean DNA value: 0.843 and standard deviation: ±0.208

* Groups A, B1 and B2 tentatively divided according to spore length, see 3.1.1.1

Table -3.8- DNA content/conidiospore nucleus in arbitrary units for isolates under investigation recorded by scanning microdensitometry (Feulgen stained nuclei).

ISOLATE	DNA CONTENT MEAN	STANDARD ERROR±	INTERNAL CONTROL	STANDARD ERROR±
GROUP A*				
130	0.950	0.020	1.550	0.016
133	0.860	0.020	1.400	0.030
140	0.950	0.020	1.550	0.016
362	0.900	0.020	1.400	0.030
G1	0.770	0.015	1.470	0.020
G16	0.690	0.020	1.400	0.030
G17	0.630	0.015	1.400	0.030
G22	0.850	0.020	1.550	0.016
GR1	0.860	0.020	1.320	0.020
111[1]	1.260	0.050	1.470	0.020
GROUP B1*				
195[2]	not recorded			
161	1.550	0.016	1.550	0.016
162[2]	not recorded			
GROUP B2*				
86207	1.450	0.020	1.400	0.030
84013	1.400	0.020	1.400	0.030
84120	1.400	0.020	1.400	0.030
G10	1.320	0.020	1.470	0.020
G18	1.310	0.027	1.320	0.020
G19[3]	0.800	0.024	1.400	0.030
G25	1.600	0.024	1.470	0.020
G23	1.730	0.030	1.470	0.020
G29	1.500	0.020	1.550	0.016
G34	1.340	0.024	1.400	0.030
GV165	1.400	0.020	1.320	0.020
GV166	1.420	0.020	1.320	0.020
GV167	1.430	0.020	1.320	0.020
GV168	1.400	0.024	1.320	0.020
GV169	1.460	0.020	1.320	0.020
GV170	1.370	0.020	1.320	0.020
F1	1.620	0.024	1.470	0.020
G334	1.420	0.017	1.470	0.020
F617	1.440	0.020	1.550	0.016
F617-7	1.430	0.026	1.400	0.030
F617-9	1.500	0.020	1.400	0.030
P671	1.300	0.020	1.320	0.020
F654-2	1.330	0.025	1.320	0.020
G859	1.500	0.020	1.550	0.016
G856	1.460	0.030	1.320	0.020
GV	1.400	0.017	1.550	0.016

* Groups A, B1 and B2 divided tentatively according to spore length, see Table-3.1-.

[1] Mean based on readings of 23 different nuclei, the value found for 111 indicates diploidy or partial diploidy.

[2] 195 and 162 were verified as diploids by Jackson and Heale and therefore were not tested again here.

[3] G19 gave DNA values corresponds to the haploid level.

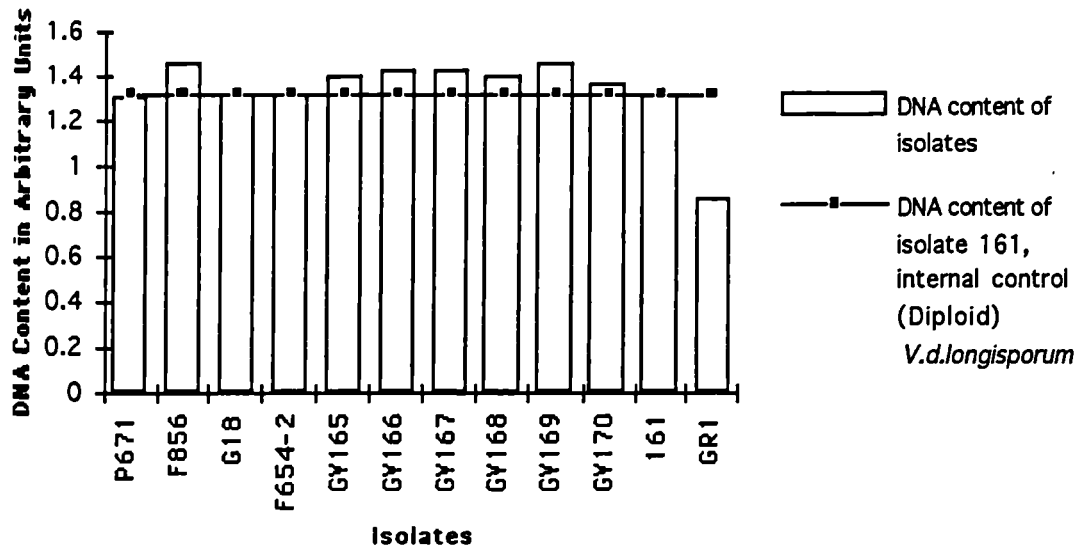


Figure-3.6a-. Graphic representation of mean arbitrary DNA content/ nucleus of 10 isolates of group B2 (P671, G856, G18, F654-2, GV165, GV166, GV167, GV168, GV169, GV170) and one isolate of group A (GR1) compared with DNA content of isolate 161, internal control, group B1 (diploid). All isolates of group B2, showed the same ploidy level as the isolate 161 of the diploid strain viz. *V.d.longisporum* with almost double the DNA content of isolate GR1 of group A of the haploid strains of *V.dahliae* . Means based on 50 individual nuclear readings /isolate (Feulgen DNA nuclei).

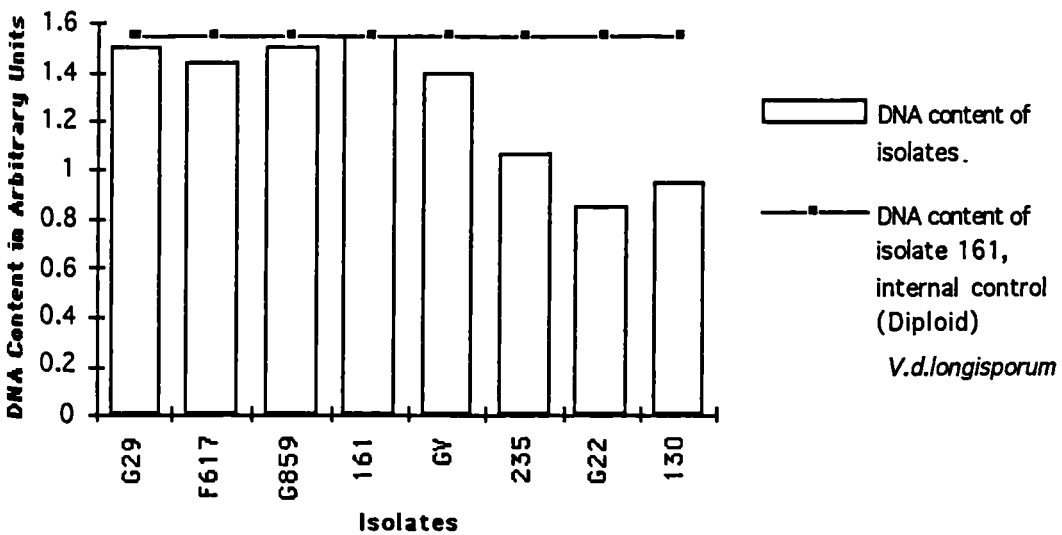


Figure-3.6b-. Graphic representation of mean arbitrary DNA content/ nucleus of 4 isolates of group B2 (G29, F617, G859, GV), one of *V.albo-atrum* (235) and 2 of group A (G22, 130) compared with DNA content of isolate 161, internal control, group B1 (diploid). All isolates of group B2, showed the same ploidy level as the isolate 161 of the diploid strain viz. *V.d.longisporum* with almost double the DNA content of isolates of group A of haploid strain of *V.dahliae* and one isolate (235) of *V. albo-atrum* (Feulgen DNA nuclei).

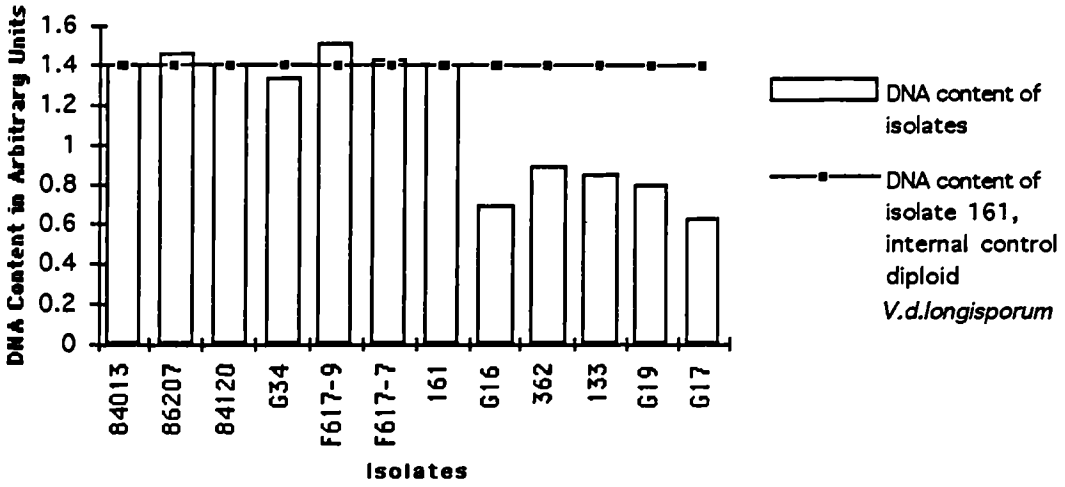


Figure-3.6c-. Graphic representation of arbitrary DNA content/ nucleus (mean based on 50 individual nuclear readings) of 7 isolates of group B2 (84013, 86207,84120, G34, F617-9, F617-7, G19), and 4 isolates of group A (G16, 362, 133, G17) compared with DNA content of isolate 161, internal control, group B1 (diploid). All isolates of group B2, except isolate G19, showed the same ploidy level as the isolate 161 of the diploid strain viz. *V.d.longisporum* with almost double the DNA content of isolates of group A of haploid strains of *V.dahliae* (Feulgen DNA nuclei).

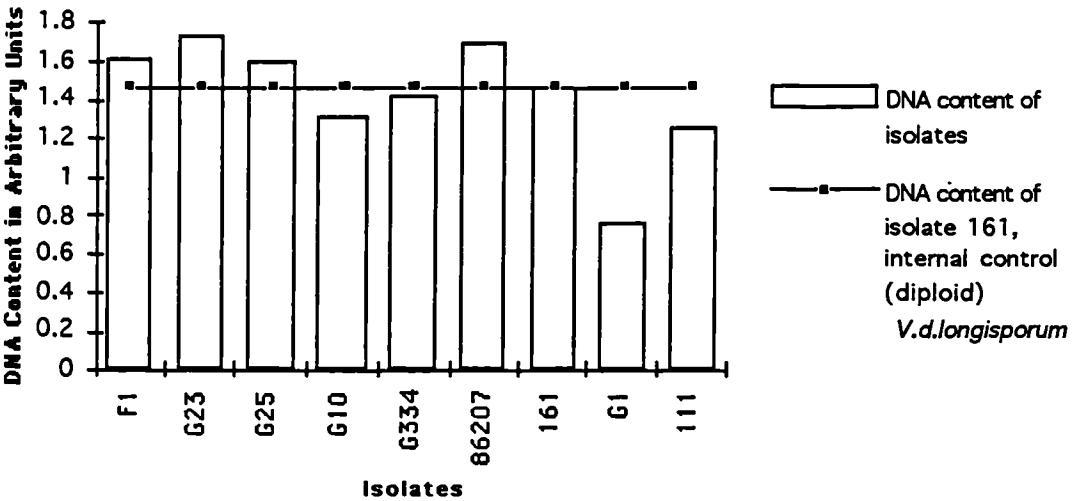


Figure-3.6d-. Graphic representation of arbitrary DNA content/ nucleus of 6 isolates of group B2 (F1,G23,G25,G10,G334,86207), and 2 isolates of group A (G1,111) compared with DNA content of isolate 161, internal control, group B1 (diploid). All isolates of group B2 and one of group A (111) showed the same ploidy level as the isolate 161 of the diploid strain viz. *V.d.longisporum* with almost double the DNA content of isolates of group A of haploid strains of *V.dahlia* (Feulgen DNA nuclei).

3.1.3. Enzymatic differences between haploid *V.dahliae* isolates and isolates of the diploid strain viz. *V.d.longisporum*

3.1.3.1.Differences in extracellular polyphenol oxidase activity (p.p.o.)

Isolates of the two groups [group A and group B (B1,B2)] were separately distinguished by comparison of their extracellular polyphenol oxidase (p.p.o.) activity, indicated by a colour reaction on a medium containing tannic acid. Discs of agar cultures for each isolate under test were taken from PDA plates and placed on Howell's medium (2.4.3.1.1.). Five days after incubation at 25 °C dark zones appeared in the medium around the discs of all the isolates of group A, except isolates 111 and G22. The dark zones represent oxidation of tannic acid and consequently indicates high levels of extracellular polyphenol oxidase activity for group A. No dark zone was visible in isolates of group B with the unique exception of isolate G19, indicating a low p.p.o. activity level in this group. Typical reactions are shown in Plate 3.9, for respective isolates of group A and group B. The results of p.p.o. activity are given in table-3.9-. Isolates G1 and G17 exhibited the most extracellular p.p.o. activity (G1 > G17 > 133 > 140 > 130 > G16) as indicated by the width and the intensity of the dark zones at the periphery of the colonies.

Table-3.9- Presence (+) or absence (-) of detectable levels of extracellular polyphenol oxidase activity by a visual plate test (2.4.3.1.1)

GROUP A*		GROUP B1*		GROUP B2*	
ISOLATE	PPO	ISOLATE	PPO	ISOLATE	PPO
130	+	195	-	86207	-
133	+	161	-	84120	-
140	+	162	-	84013	-
362	+			G10	-
G1	+			G18	-
G16	+			G19	+
G17	+			G25	-
G22	-			G23	-
GR1	+			G34	-
111	-			GV165	-
				GV166	-
				GV167	-
				GV168	-
				GV169	-
				GV170	-
				F1	-
				G334	-
				F617	-
				F617-7	-
				F617-9	-
				P671	-
				F654-2	-
				G856	-
				GV	-
				G859	-

* Groups A, B1 and B2 tentatively divided according to spore length, see Table -3.1-

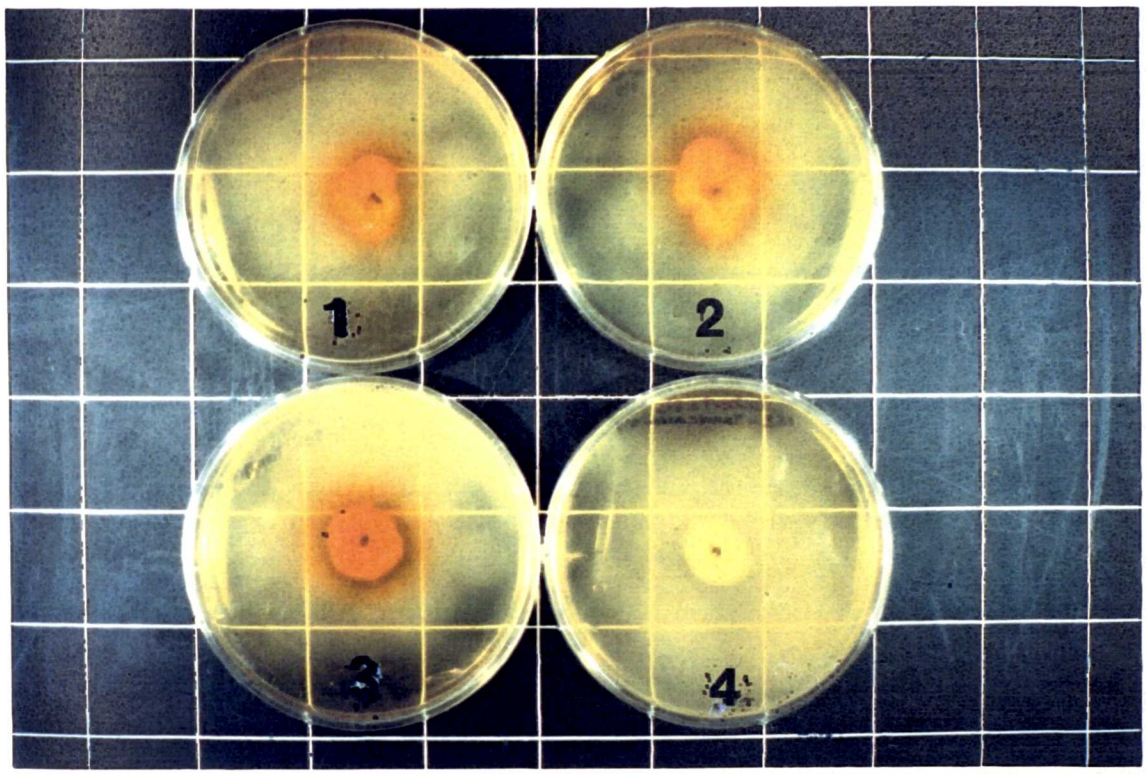


Plate-3.9- Qualitative comparison of extracellular polyphenol oxidase activity on modified Howell's medium between isolates of group A (1: Isolate 140, 2: Isolate 130, 3: Isolate 133) and one of group B1 (4: 162). Isolates of the group A exhibited a dark zone, representing activity of the enzyme, while isolates of the B1 group showed no dark zone indicating low extracellular p.p.o activity.

3.1.3.2. Differences in polygalacturonase (p.g.) activity

Isolates of the two groups were tested for extracellular p.g. activity, indicated by clear zones around colonies on a medium containing polygalacturonic acid. Discs of agar cultures for each isolate under test were taken from PDA plates and placed on Howell's medium (2.4.3.1.2.). Six days after incubation, cultures were flooded with 1N HCl and the extent of clear zones around the colonies indicated their polygalacturonase activity. All isolates, irrespective of the group to which they belonged, exhibited p.g. activity. Four isolates of group A (362, GR1, 130, 133) and two of the sub-group B1 (195, 161) exhibited more p.g. activity as compared with all other isolates in the groups. The results of p.g activity for all isolates under test are given in table-3.10.-.

Table-3.10- Extracellular polygalacturonase activity (+,++) by a plate-clearing test (2.4.3.1.2.).

GROUP A*		GROUP B1		GROUP B2	
ISOLATE	P.G	ISOLATE	P.G	ISOLATE	P.G
130	++	195	++	86207	+
133	++	161	++	84120	+
140	+	162	+	84013	+
362	++			G10	+
G1	+			G18	+
G16	+			G19	+
G17	+			G25	+
G22	+			G23	+
GR1	++			G34	+
111	+			GV165	+
				GV166	+
				GV167	+
				GV168	+
				GV169	+
				GV170	+
				F1	+
				G334	+
				F617	+
				F617-7	+
				F617-9	+
				P671	+
				F654-2	+
				G856	+
				GV	+
				G859	+

* Groups A, B1 and B2 tentatively divided accordins to spore length, see Table-3.1.-.

++ Represents more p.g activity as indicated by the width of the clear zone (> 1cm) around the colony.

3.1.4. Sensitivity to UV light

Only preliminary UV sensitivity tests were carried out with selected isolates of the three groups. Two representative isolates of group A (130, G1), one isolate from group B1 (161) and one isolate of group B2 (G334) were subjected to UV light treatment and their survival curves were plotted. Preliminary experiments showed that to obtain consistent results, it was necessary to spread the appropriate spore suspension (100 spores/ plate) on the entire surface of the CM plate first and then to irradiate for the appropriate time. Dilution of the spore suspension after the irradiation resulted in experimental error and inconsistent results. The UV dosage was up to 180 sec at 20 sec intervals; the % viability was determed by comparing the number of spores that formed visible colonies after irradiation and colonies from control, non-irradiated spores. Figure -3.7- shows the survival curves for all four isolates tested . In these preliminary tests, the three groups were not clearly distinguished by their sensitivity to UV light, although isolate G1 of group A of haploids seemed to be less sensitive than the three other isolates tested here. No further experiments involving UV irradiation were performed.

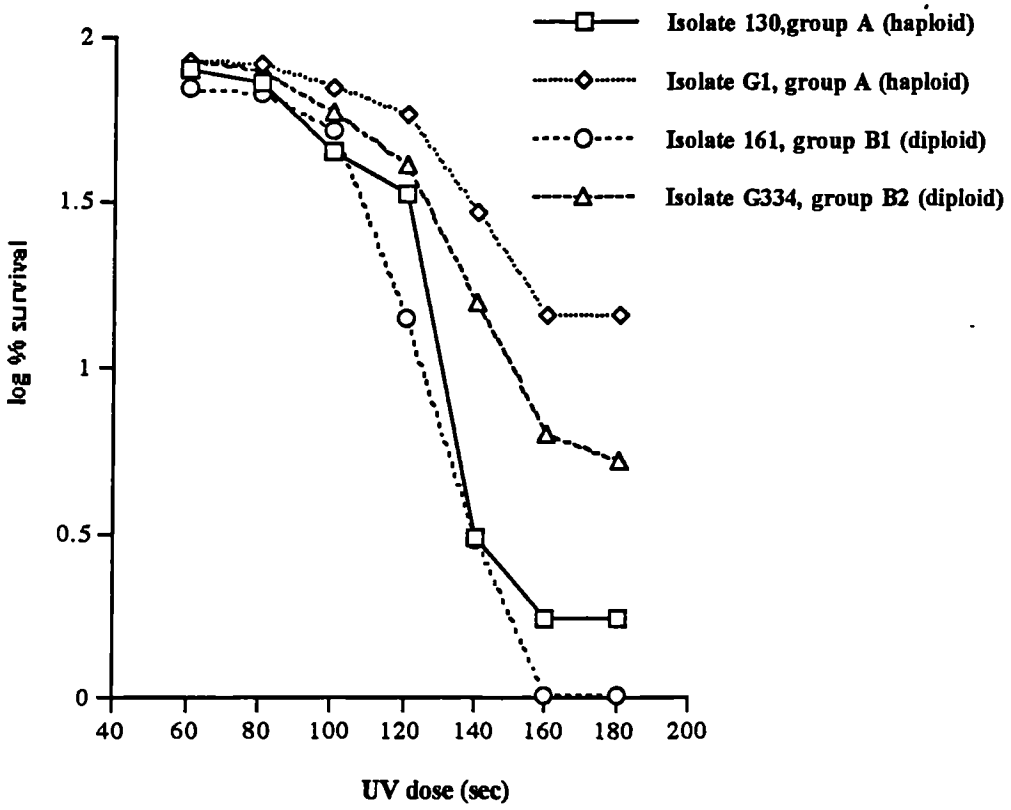


Figure-3.7- Survival curves of representative isolates of the three groups irradiated with UV light.

3.1.5. Molecular analysis

3.1.5.1. Genetic fingerprinting of isolates of *V.dahliae* (haploid), *V.d.longisporum* (diploid) and *V.albo-atrum* (haploid) by Random Amplified Polymorphic DNA (RAPDs)

To further characterize and distinguish the isolates of the haploid strains of *V.dahliae* (Group A) from isolates of the diploid strain viz. *V.d.longisporum* (group B) at the molecular level, their genomic DNA was amplified using single short nucleotide primers (table -2.4-), i.e. 10mers and 20mers, at low annealing temperatures as described in section 2.5.2.4. Low annealing temperatures (35^0C, 37^0C) produce some mismatching between primer and template DNA, thus generating multiple bands.

To select which primers generated informative profiles, 15 randomly chosen synthetic nucleotides (supplied by OPERON) and 5 primers based on the intergenic spacer region of the ribosomal RNA (rRNA) gene complexes of *Penicillium hordei* (table -2.4-) were used to amplify genomic DNA from two isolates (haploid isolate G1 of *V.dahliae* from group A and isolate G10 of the diploid pathogenic strain viz. *V.d.longisporum* from group B). The RAPD patterns obtained differed markedly according to which of the primers tested that resulted in amplification. Some of the primers generated more DNA fragments than did others, and others failed to produce any fragments.

Of the 20 primers tested in total, only 3 (OPA13 from OPERON laboratories and P2, P4A based on the IGS region of *P.hordei*) produced clear informative amplification patterns which appeared to have consistent, discriminating characteristics; these were further used to amplify the genomic DNA of all isolates given in table -3.1-.

Up to 37 amplified bands (between 0.4 and 2.35kb) were generated in total (for all isolates and primers). Primer P4 generated 9 bands in total, primer P2:17 bands and primer OPA13:11 bands.

Amplification with primer P2 (Table-3.12-, Plate-3.10-) generated two distinct bands of 1.05kb and 0.65kb in (i) all typical isolates of the diploid *V.d.longisporum* group B (including the two putative recombinants 195, G19) and (ii) in Group A only in G22, a putative recombinant *V.d.longisporum* isolate, that had been provisionally placed in this group . These two bands were not present in the other 8 haploid *V.dahliae* Group A isolates or the other putative recombinant isolate *V.d.longisporum* 111 provisionally placed in Group A, but isolate 234 of *V.albo-atrum* from lucerne also generated this 0.65 kb band. Isolate G22 (a putative recombinant of *V.d.longisporum*) additionally gave a 1.5 kb band that produced elsewhere only by the 234 isolate of *V.albo-atrum*. Isolate 111 (a putative recombinant of *V.d.longisporum*) generated the same amplification pattern as all the typical haploid isolates of group A, with the one addition of a 1.1kb band that was only found elsewhere in both *V.albo-atrum* isolates tested here. These two isolates (*V.albo-atrum*) gave 5 distinct bands (see table-3.13-). There were no bands which were unique to Group A normal haploids which were not found in the other isolates/groups of *V.d.longisporum*. Putative recombinants of *V.d.longisporum* generated some bands which were also common (i)to either "normal" diploids isolates of Group B, (ii) to haploid isolates of Group A and (iii) also to one or both *V.albo-atrum* isolates. The summary of the RAPDs amplification patterns obtained with P2 are given in table-3.12- and the common bands among the groups obtained with primer P2 are given in table -3.13-.

Amplification with primer P4a (Plate-3.11- , Table-3.14-) gave rise to a band of 1.40kb in (i) all

isolates of diploid *V.d.longisporum* Group B,(ii) in all putative recombinants of *V.d.longisporum*, (isolate 111 initially placed in group A, G19 and 195 of group B) except of the isolate G22, haploid *V.dahliae* Group A; and (iii) one isolate, 234 (haploid), of *V.albo-atrum* from lucerne. All 'normal' 8 haploid isolates of group A and the 230 isolate of *V.albo-atrum* from chrysanthemum failed to generate this 1.40kb band. A band of 1.28kb was observed only in (i) haploid isolates of group A (except for putative recombinant isolate 111), (ii) in two putative recombinants placed in group B (195 and G19) and (iii) the two haploid *V.albo-atrum* isolates. *V.albo-atrum* isolates . generated two unique bands (2.35kb and 1.50kb) that were not found in any other group (see table-3.15-). There were no bands unique to Group A *V.dahliae* haploids. Putative recombinant isolates of *V.d.longisporum* generated bands common to (i) isolates of the Group A haploids,(ii) to Group B *V.d.longisporum* diploids and (iii) to *V.albo-atrum* isolates. The summary of the RAPD amplification patterns with primer P4a are given in table -3.14-. and the common bands among the groups obtained with P4a are given in table-3.15-.

Table-3.11-Isolates of *V.dahliae*, *V.d.longisporum* and *V.albo-atrum* used for RAPDs employing primers P2, P4a & OPA13 (see Table-2.4- for sequence of primers).

GROUP A* *V.dahliae* (haploid)	HOST	GROUP B* *V.d.longisporum* (diploid)	HOST	*V.albo atrum* (haploid)	HOST
G1	Clover	161	Sugar beet	230	Chrysanthemum
362	Shepherd's purse	162	Rape	234	Luceme
130	Tomato	195#	Horse radish		
133	Tomato	G19#	Rape		
C13	Cotton	G29	Rape		
G16	Potato	G10	Rape		
G22#	Rape	G34	Rape		
140	Eggplant	GV167	Rape		
G17	Stock	G18	Rape		
111#	Brussels sprouts	G23	Rape		
		G25	Rape		
		84013	Chinese cabbage		
		84120	Chinese cabbage		
		86207	Wild radish		
		F617-9	Rape		
		F617-7	Rape		
		P671	Rape		
		F654-2	Rape		
		G334	Rape		
		G859	Rape		
		S856•	Rape		

* Group tentatively divided according to spore length (1.1.1.2)

#Putative recombinants based upon previous results showing recombination for diploid- associated characters in these isolates.

• S856=G856, an isolate from Senicerande, Germany

Amplification with the random primer OPA13 (Table-3.16-, -3.17-, Plate-3.12-) generated four distinct bands of 1.55, 1.35, 0.95 and 0.70kb only (i) for isolates of group B (including the two putative recombinants G19 and 195 initially placed in group B) and (ii) one putative isolate 111 of group A. Isolates of group A generated two bands of 1.45 and 1.22 kb that were found in only one isolate of Group B (isolate S856). Putative recombinant isolates (G19, 195 and G22) and S856 of group B had intermediate patterns. The two isolates of *V.albo-atrum* generated the 0.95kb band that was only observed for all isolates of Group B and the 3 putative recombinant isolates 111 of group A, G19 and 195 of group B. Isolates of *V.albo-atrum* generated 2 distinct bands of 0.85kb and 0.60kb that were not found in any other Verticillium isolate tested here. The summary of the RAPD amplification patterns generated with primer OPA13 is given in table -3.16- and the common bands among the groups obtained with OPA13 are given in table-3.17-.

Table -3.12- RAPD bands obtained with primer P2. (Sizes are given in kbs).

V.dahliae GROUP A haploids (n) 8 isolates	*V.d.longisporum* GROUP B diploids (2n) 18 isolates	S856 GROUP B (2n)	195# (2n)	G19# (n)	G22# (n)	111# (2n)	*V.a.a** 230 (n)	*V.a.a** 234 (n)
					1.5			**1.5**
							1.40	
1.35	1.35	1.35	1.35	1.35	1.35	1.35		1.35
							1.25	
1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	
								1.15
						1.10	**1.10**	**1.10**
	1.05	**1.05**	**1.05**	**1.05**	**1.05**			
0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	
								0.85
0.75	0.75	0.75	0.75	0.75	0.75	0.75		
	0.65	**0.65**	**0.65**	**0.65**	**0.65**			**0.65**
0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
								0.48
0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
0.40	0.40	0.40	0.40	0.40	0.40	0.40		0.40

#Putative recombinants of *V.d.longisporum*.

Bands in bold type are distincive bands among the different groups.

* *V.albo-atrum*

Table-3.13- Summary of the common RAPD bands in distinct groups obtained with primer P2 (Sizes are given in kb).

Bands only found in haploids (*V.d.*) and *V.d.l.* recombinants	-	-	-	-	-	-	-
Bands only found in *V.d.l.* diploids and putative recombinants of *V.d.l.*	1.05	-	-	-	-	-	-
Bands only found in putative recombinants of *V.d.l.* and one or other *V.a.a.* isolate	1.10	1.50	-	-	-	-	-
Bands only found in *V.d.l.* diploids, putative recombinants of *V.d.l.* and one or other *V.a.a.* isolate.	0.65	-	-	-	-	-	-
Bands only found in one or other *V.a.a.* isolates.	0.48	0.85	1.15	1.25	1.40	-	-
Bands only found in haploid *V.d.*, *V.d.l.* diploids, and putative recombinants of *V.d.l.*	0.75	-	-	-	-	-	-
Bands common to haploid *V.d.*, *V.d.l.* diploids, putative recombinants of *V.d.l.* and one or other *V.a.a.* isolates	0.40	0.45	0.50	0.60	0.95	1.20	1.35

V.d.l=V.d.longisporum

V.d.=V.dahliae

V.a.a =V.albo-atrum

Table -3.14.-Summary of the RAPD bands obtained with primer P4a. Sizes are given in kb.

V.dahliae **Group A haploids (n) (8 isolates)**	***V.d.longisporum*** **Group B diploids (2n) (18 isolates)**	**S856 Group B (2n)**	**195# (2n)**	**G19# (n)**	**G22# (n)**	**111# (2n)**	**230 *V.a.a* (n)**	**234 *V.a.a* (n)**
								2.35
2.00	2.00	2.00	2.00	2.00		2.00		
1.93	1.93	1.93	1.93	1.93		1.93		
								1.50
	1.40	**1.40**	**1.40**	**1.40**		**1.40**		**1.40**
1.28			**1.28**	**1.28**	**1.28**		**1.28**	**1.28**
		1.10	1.10	1.10				
0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	

#Putative recombinants. Band 1.84kb was only generated by 4 diploids [GV167, G18,84013,G859].

Bands in bold type are distinctive bands among the groups. *V.a.a =V.albo-atrum*

Table-3.15- Summary of the common RAPD bands in distinct groups obtained with P4a. Sizes are given in kb.

Bands only found in haploids (*V.d.*) and putative recombinants of *V.d.l*	-	-
Bands only found in *V.d.l*:diploids and putative recombinants of *V.d.l*	1.10	-
Bands only found in putative recombinants of *V.d.l* and one or other *V.a.a.* isolates.	-	-
Bands only found in haploid *V.d.* and recombinants recombinants of *V.d.l.* and one or other *V.a.a.* isolates.	1.28	-
Bands only found in *V.d.l* and recombinants and one or other *V.a.a.*	1.40	-
Bands only found in one or other *V.a.a.* isolates.	2.35	1.50
Bands only found in haploid *V.d.* *V.d.l.* : diploid and putative recombinants of *V.d.l.*	2.0	1.93
Bands common to haploid *V.d.*, *V.d.l.*:diploids, putative recombinants of *V.d.l.* and one or other *V.a.a.* isolates.	0.90	-

Table-3.16- Summary of the amplification patterns obtained with primer OPA13. Sizes are given in kbs

V.dahliae **Group A haploid (n) 8 isolates**	***V.d.longisporum*** **Group B diploid (2n) 18 isolates**	**S856** **2n**	**195#** **2n**	**G19#** **n**	**G22#** **n**	**111#** **2n**	***V.a.a*** **230** **n**	***V.a.a*** **234** **n**
		1.80						
	1.55	**1.55**	**1.55**	**1.55**		**1.55**	**1.55**	
1.45		**1.45**			**1.45**			
	1.35					1.35		
1.22		**1.22**	**1.22**	**1.22**	**1.22**			
1.05	1.05	1.05	1.05	1.05	1.05	1.05		
	0.95		**0.95**	**0.95**		**0.95**	**0.95**	**0.95**
								0.85
0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	
	0.70	**0.70**	**0.70**	**0.70**		**0.70**		**0.70**
							0.60	

#Putative recombinants. Bands in bold type are distinctive bands among the groups. - none

Table-3.17- Summary of the common RAPD bands in distinct groups obtained with primer OPA13. Sizes are given in kb.

Bands only found in haploids (*V.d.*) putative recombinants of *V.d.l* and isolate S856.	1.25	1.45	-
Bands only found in diploids:*V.d.l.* and putative recombinants of *V.d.l.*	1.35	-	-
Bands only found in putative recombinants of *V.d.l.* and one or other *V.a.a.* isolate.	-	-	-
Bands only found in haploid *V.d.* and putative recombinants of *V.d.l.* and one or other V.a.a. isolate.	-	-	-
Bands only found in *V.d.l*:diploids, putative recombinants of *V.d.l.* and one or other *V.a.a* isolate..	1.55	0.95	0.70
Bands only found in one or other *V.a.a.* isolate.	0.85	0.60	-
Bands only found in haploid *V.d.*, *V.d.l.*:diploids and putative recombinants of *V.d.l.*	1.05	-	-
Bands common to haploid *V.d.*, *V.d.l.*:diploids, putative recombinants of *V.d.l.* and one or other *V.a.a.* isolate.	0.75	-	-

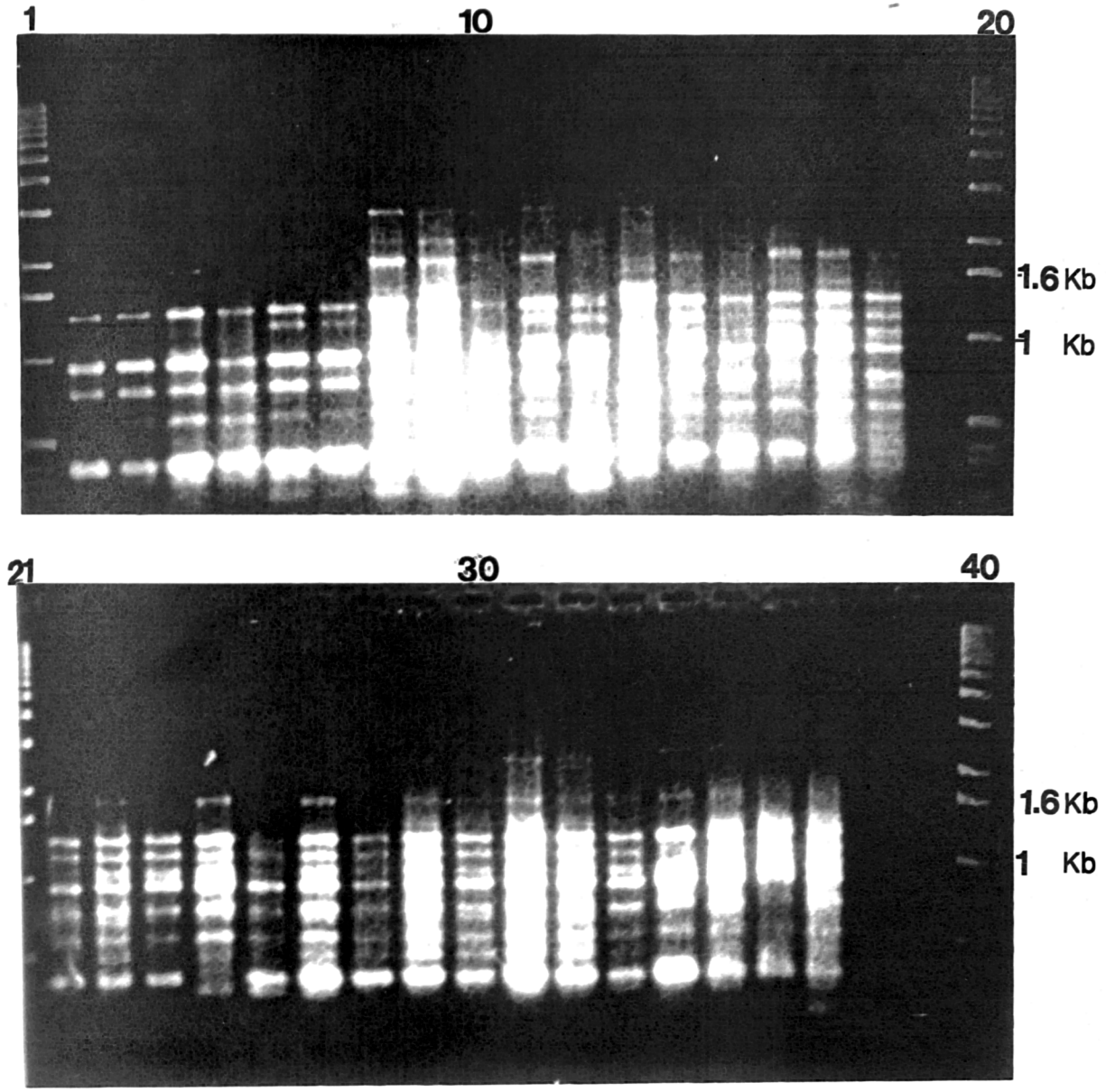


Plate-3.10- .Random Amplified Polymorphic DNA (RAPD) patterns (generated with primer P2) of Group A isolates of the haploid strains of *V.dahliae* (lanes 2-11), of Group B isolates of the diploid strain viz *V. d longisporum* (lanes 14, 16-18 and 22- 37) and two isolates of *V.albo-atrum* .Lanes 4, 9 ,16 and 17 are for the putative recombinant isolates 111, G22, 195 and G19 respectively. Lanes 1, 20, 21 and 40 contain molecular weight markers (1kb DNA ladder). Lanes 2, 3, 4 , 5, 6, 7, 8, 9,10, 11, 15 are for isolates G1, 362, 111,130, 133, C13, G16, G22, 140, G17 and 362 respectively of the haploid strains of *V.dahliae* (group A). Lanes 12 and 13 are for isolates 230 and 234 of *V.albo-atrum.* Lanes 14, 16, 17, 18, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36,37 are for isolates 161, 195, G19, G29, G10, G34, GV167, G18, G23, G25, 84013, 84120, 86207, F617-9, P671, F654-2, G334, G859 and S856 respectively of the diploid strain of *V.d.longisporum* (group B). DNA was fractionated on a 1.5% agarose gel and stained with ethidium bromide.

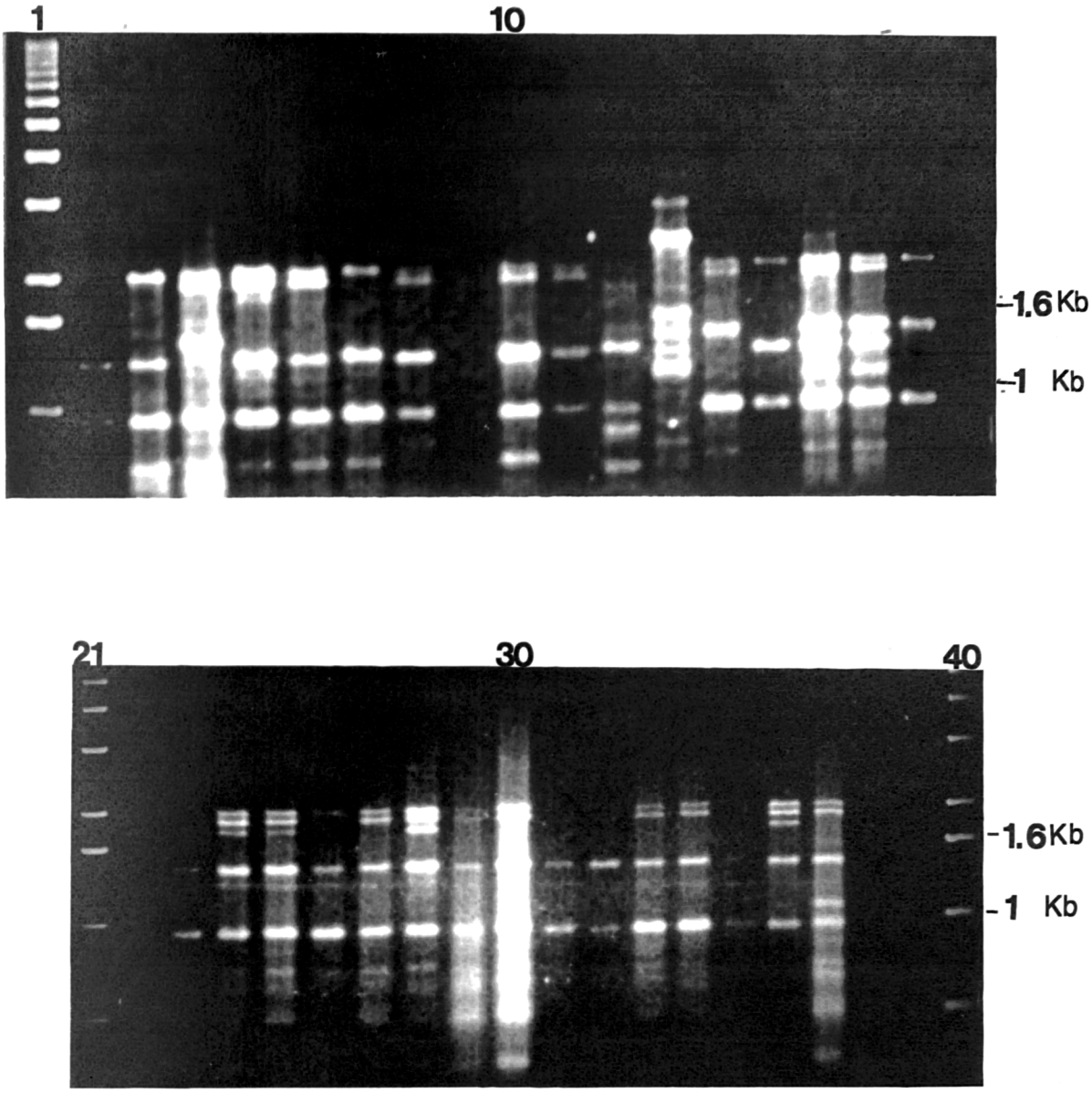


Plate-3.11- .Random Amplified Polymorphic (RAPD) DNA patterns (generated with **primer P4a** of Group A isolates of the haploid strains of *V.dahliae* (lanes 2-11), of Group B isolates of the diploid strain viz *V. d longisporum* (lanes 14, 16-18 and 22- 37) and two isolates of *V.albo-atrum*. Lanes 4, 9 ,16 and 17 are for the putative recombinant isolates 111, G22, 195 and G19 respectively. Lanes 1, 20, 21 and 40 contain molecular weight markers (1kb DNA ladder). Lanes 2, 3, 4 ,5, 6, 7, 8, 9,10, 11, 15 are for isolates G1, 362, 111, 130, 133, C13, G16, G22, 140, G17 and 362 respectively of the haploid strains of *V.dahliae* (group A). Lanes 12 and 13 are for isolates 230 and 234 of *V.albo-atrum*. Lanes 14, 16, 17, 18, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36,37 are for isolates 161, 195, G19, G29, G10, G34, GV167, G18, G23, G25, 84013, 84120, 86207, F617-9, P671, F654-2, G334, G859 and S856 respectively of the diploid strain of *V.d.longisporum* (group B). DNA was fractionated on a 1.5% agarose gel and stained with ethidium bromide.

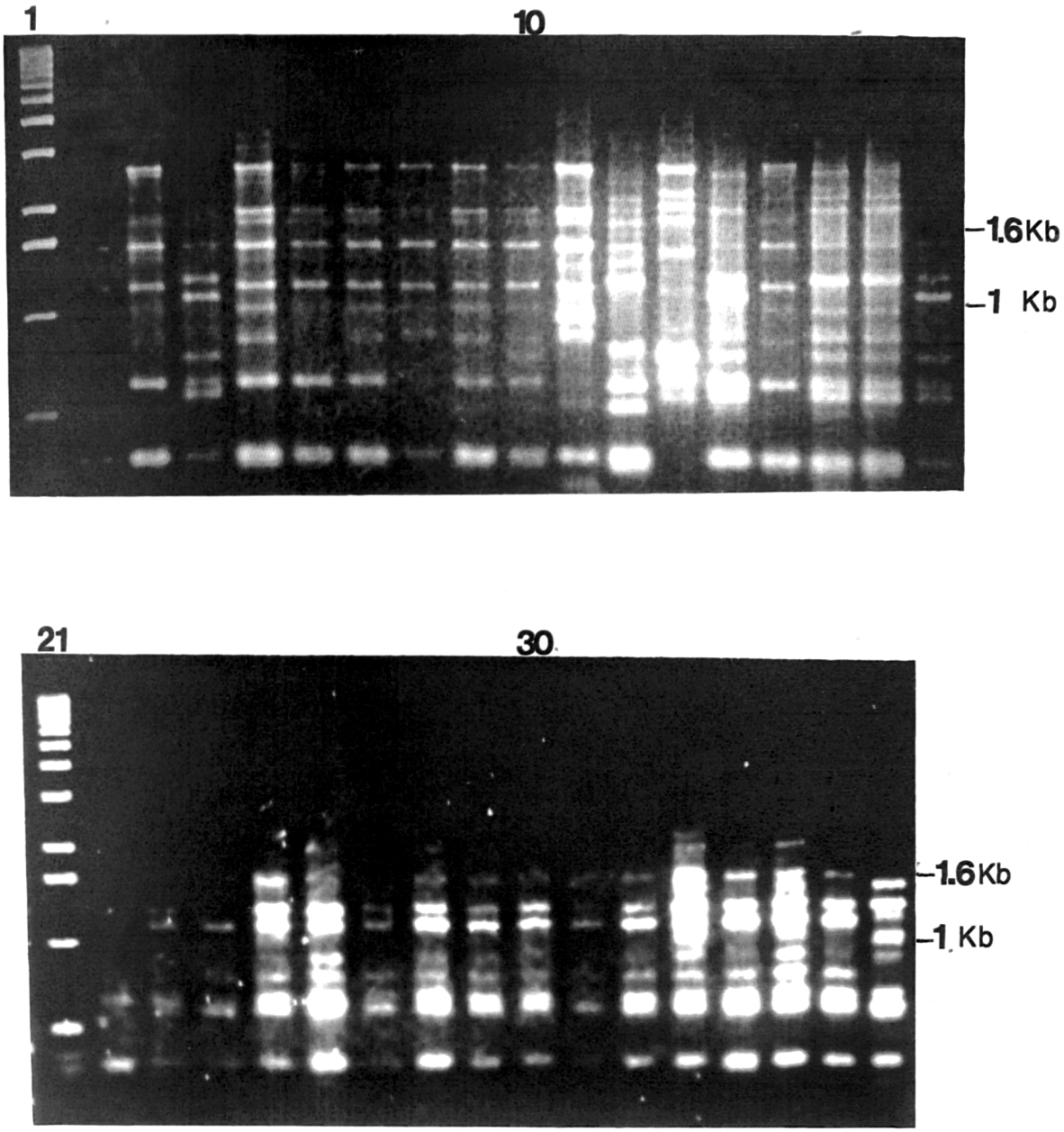


Plate-3.12- .Random Amplified Polymorphic DNA(RAPDs) patterns (generated with **primer 0PA13)** of Group A isolates of the haploid strains of *V.dahliae* (lanes 2-11), of Group B isolates of the diploid strain viz *V. d longisporum* (lanes 14, 16-18 and 22- 37) and two isolates of *V.albo-atrum.* Lanes 4, 9 ,16 and 17 are for the putative recombinants isolates 111, G22, 195 and G19 respectively. Lanes 1, 20, 21 and 40 contain molecular weight markers (1kb DNA ladder). Lanes 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 15 are for isolates G1,362, 111,130, 133, C13, G16, G22, 140, G17 and 362 respectively of the haploid strains of *V.dahliae* (group A). Lanes 12 and 13 are for isolates 230 and 234 of *V.albo-atrum.* Lanes 14, 16, 17, 18, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36,37 are for isolates 161, 195, G19, G29, G10, G34, GV167, G18, G23, G25, 84013, 84120, 86207, F617-9, P671, F654-2, G334, G859 and S856 respectively of the diploid strain of *V.d.longisporum.*(group B). DNA was fractionated on a 1.5% agarose gel and stained with ethidium bromide.

To compare the DNA profiles generated for each isolate for all three primers, the data were converted to 0 or 1 if a particular band was absent or present respectively. All the data for the 37 different bands and for the 33 isolates that were used were combined together and were analysed using an average-linkage cluster analysis (Unweighted Pair-Group Method with Arithmetic Averages,UPGMA) with Jaccard's coefficient as described in section 2.4.5.5.

A dendrogram derived using UPGMA analysis separated the isolates into four distinct main clusters (Figure-3.8a-). The first cluster consisted of 8 isolates of haploid strains of *V.dahliae* (proved earlier in this investigation to be haploids by Feulgen microdensitometry), irrespective of the host from which they originated (G1, 362, 130, 133, 140, C13, G17, and G16). The second main cluster consisted of 19 isolates of the diploid strain of *V.d.longisporum* (G10, GV167, 84013, G859, G18, 161, 162, G34, G25, 84120, 86207, P671, F654-2, G334, G29, G23, F617-9, F617-7, S856 and two putative recombinants viz. 195 and G19 (all in Group B) and the one putative recombinant isolate of Brussels sprouts (111) that had been placed provisionally in group A (this isolate produced short spores but was subsequently proved to be diploid by Feulgen microdensitometry). All isolates of this diploid cluster, except the putative recombinant G19,(which can be seen to be in an individual sub-cluster with putative recombinant isolate 195) were previously shown to be diploids by Feulgen microdensitometry (3.1.2.2). The third and fourth cluster consisted of the two isolates of the separate species *V.albo-atrum.* (230, 234). [The two different *V.albo-atrum* isolates, one from chrysanthemum (230) and one from lucerne (234) formed two different clusters from each other.]

When isolate G22 (in the first cluster) was first isolated, it was observed to produce long spores (8.70±0.23), but after two years in culture it produced only short spores(4.607±0.061) although it still retained one of the characters of the diploid strains, viz. the absence of detectable extracellular polyphenol oxidase (p.p.o) activity. In this first cluster of the haploids G22 isolate was somehow separated, forming a sub-cluster (being less similar to the other haploids). Similarly, one isolate from clover(G1) and one isolate from potato (G16) isolated in Germany were less similar to the other haploid isolates, forming distinct sub-groups.

The putative recombinant isolate 111 had been isolated from a Brassica crop (viz. Brussels sprouts) in the UK in 1957 and was still diploid by the Feulgen criterion as well as by absence of detectable extracellular P.p.o activity. It clustered in the same group as the rest of the diploids, but was nevertheless in its own sub-group.

The two other putative recombinants viz. isolate 195 (*V.d.longisporum*) and isolate G19 formed another sub-group in the main diploid cluster; they both produced long spores and were provisionally placed in Group B. G19 was haploid by the Feulgen criterion and also the presence of detectable extracellular P.p.o activity. 195 had all the diploid characters except the shape of microsclerotia that were as compact as the haploid strains of group A. The isolate S856=G856 from Sennicerande (Germany) appeared to be a normal diploid except of its distinct RAPD pattern that could be due to it being a geographical variant.

The two isolates of *V.albo-atrum* formed two distinct clusters and were quite distinct from the diploid isolates of *V.d.longisporum* as well as isolates of the haploid strains of *V.dahliae*. *V.albo-atrum* from lucerne is known to be a distinct *V.albo-atrum* pathotype with particular characters associated (e.g.

optimum temperature growth 25-26 °C as compared with the 22-23 °C for all other *V.albo-atru* including the *V.albo-atrum* chrysanthemum).

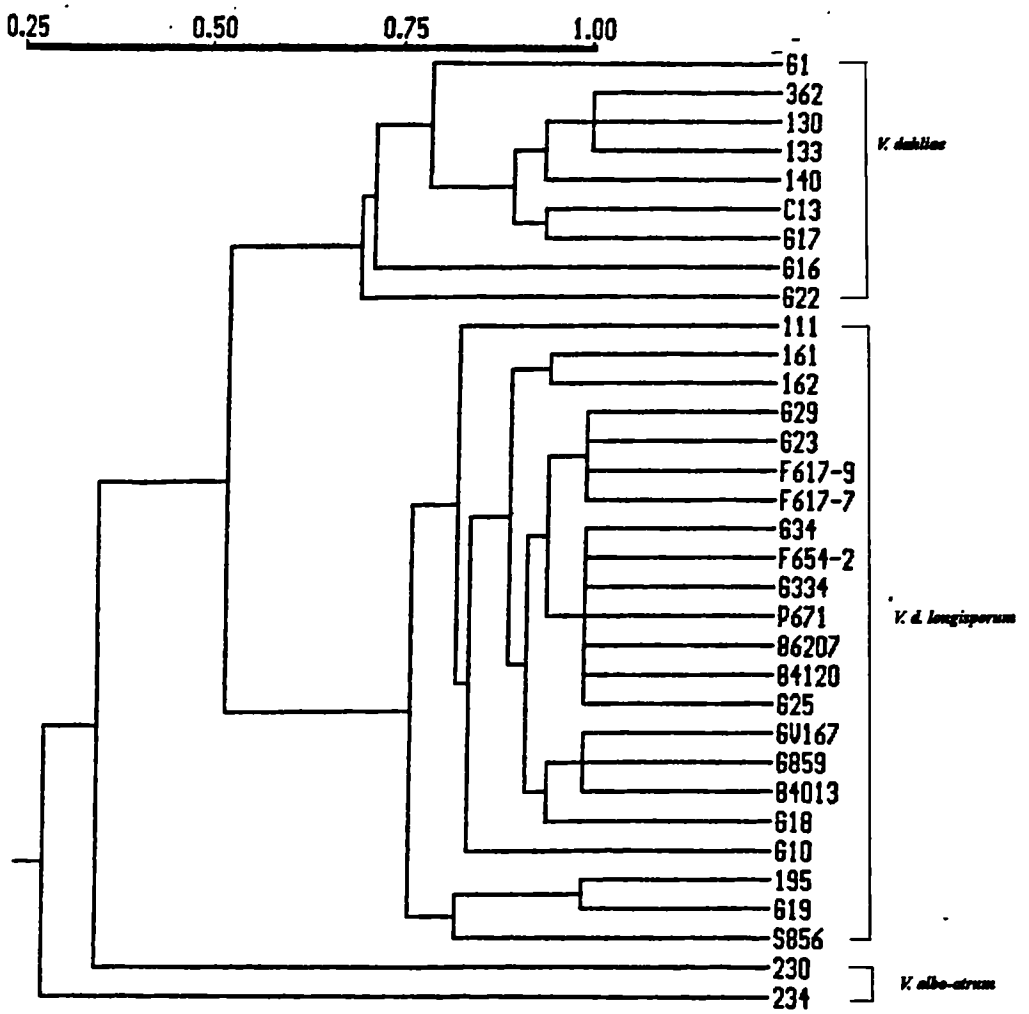


Figure-3.8a- Dendrogram derived from RAPDs analysis obtained using three primers (P2, P4a and OPA13) using UPGMA, showing relationships among 8 isolates of the haploid strains of *V.dahliae*, 19 isolates of the diploid strain viz. *V.d.longisporum* (including S856), 4 putative recombinants of *V.dahliae longisporum* and two isolates of *V.albo-atrum*. Top scale is percentage similarity using Jaccard's similarity coefficient (2.4.5.5).

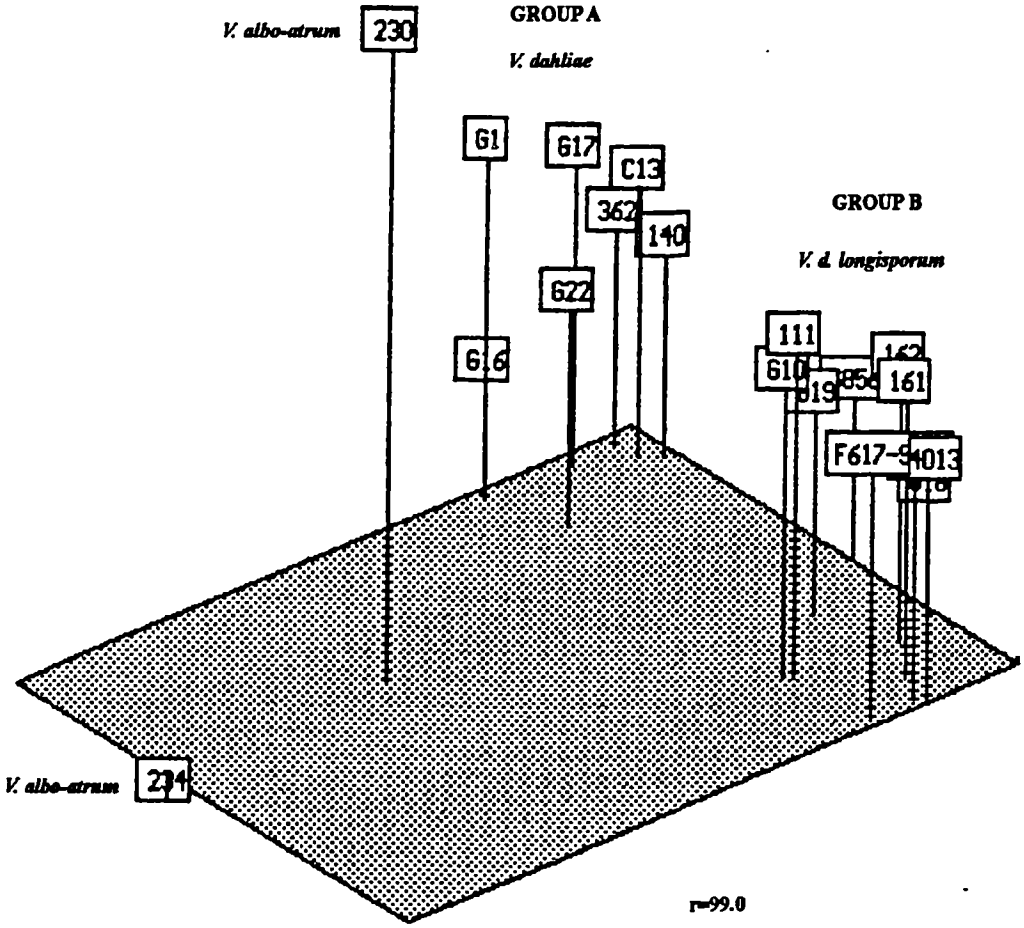


Figure -3.8b.- Principle co-ordinate analysis of RAPDs data obtained using 3 primers (P2, P4a and OPA13). The description of the isolates is given in table-3.11.-. The ploidy of all 8 isolates in the haploid cluster was confirmed by Feulgen microdensitometry and were all in group A. The ploidy of all 19 isolates in the diploid cluster was confirmed by Feulgen DNA microdensitometry. The 3 putative recombinant isolates (111, G19 and 195) were in the main group of diploids and the putative recombinant G22 was in the group of haploids.

3.1.5.2 Chromosome-length DNA polymorphism among strains of *V.dahliae*, *V.d.longisporum* and the four putative recombinants of *V.d.longisporum*.

The isolates of *Verticillium* species under study were classified into three main groups (Group A of haploid strains, Group B of diploids and Group R of the four recombinants) according to the previous characters tested (length of spore, shape of microsclerotia, extracellular p.p.o, ploidy levels as detected by Feulgen microdensitometry, and RAPDs patterns generated by three different primers).

To test whether variation in electrophoretic karyotype was correlated with the three above groups (as defined by the characters tested), the electrophoretic karyotypes, of 8 isolates (2 isolates from the Group A :haploids G17,140; 2 from group B: diploids 161, G859; and from Group B:4 putative recombinants: G22, 111, G19, 195) were compared by separation of chromosome-length DNA by PFGE-CHEF electrophoresis (2.4.6.3.).

To obtain chromosomal DNA as intact as possible, protoplasts were obtained from 48h germinated conidia. Protoplasts were produced from young hyphae by incubation in a lysing solution as described in section 2.4.6.1; they were first observed after one hour incubation emerging from the apical regions of young hyphae. The time required for the completion of the hyphal wall digestion was 2.5h (for 100mg of mycelia).

The shape of protoplasts (for all isolates under test) varied from spherical to oval and the size ranged from 2.2µm to 7.7µm (see Plate-3.13-). Table-3.18- gives the mean diameter in µm for all isolates under test. There was no significant difference in the diameter of protoplasts among the three different groups.

Table-3.18- Mean protoplast diameter (µm) of 50 protoplasts tested per isolate.

ISOLATE	161 Group B	G859 Group B	G17 Group A	140 Group A	111 Group R	G22 Group R	G19 Group R	195 Group R
MEAN	4.224	5.258	5.214	5.140	5.962	4.73	5.100	5.06
STANDARD ERROR	0.158	0.126	0.146	0.110	0.203	0.148	0.120	0.153

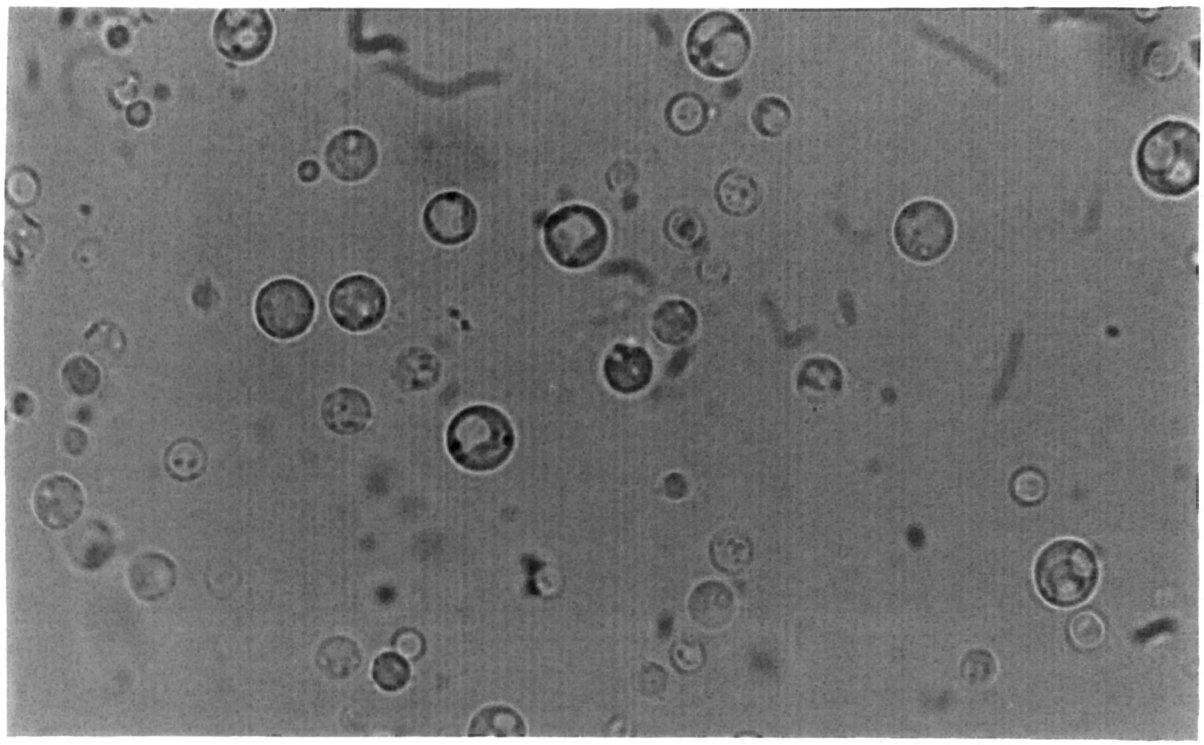


Plate-3.13- Protoplasts obtained from young hyphae of *V.d.longisporum*, isolate 161 (diploid) after 2h digestion in lysing solution. The size of protoplast diameter differed, ranged between 2.2 µm and 7.7 µm. Protoplasts were viewed under a light microscope (X 1250).

Chromosomal DNA from the above isolates was released from hyphal protoplasts in agarose plugs as described in section 2.4.6.2. and subjected to PFGE-CHEF electrophoresis (2.4.6.3.). The migration rate of DNA molecules through agarose gels is determined by agarose concentration, voltage, pulse time and running time. In preliminary experiments, *Verticillium* DNAs were electrophoresed for 168h but this was not enough to fractionate shorter chromosomal DNAs (data not shown). In order to improve the DNA separation, the running time was increased to 264h with switching intervals gradually decreasing from 3600s to 1000s.

A large degree of chromosome-band polymorphism was observed. No two isolates had exactly the same karyotype (Plate-3.14-.). Table-3.19- gives the actual size in Mbs of the chromosome-size DNA fractionated by PFGE-CHEF. Diploid isolate 161 of *V.d.longisporum* resolved 9 (or 10) bands whereas haploid isolates G17 and 140 resolved 5 (or 6) and 4 bands respectively. Putative recombinants of *V.d.longisporum*: isolate 195, (diploid) produced 8-10 bands; isolate 111 (diploid) resolved 9 bands, and isolate G22 (haploid) resolved 7 bands. Furthermore diploid strains produced more chromosome bands (9-10) than the haploids strains (4-7) under test. The chromosome-length DNA of the other two isolates G859 (diploid) and G19 (putative recombinant) could not be resolved.

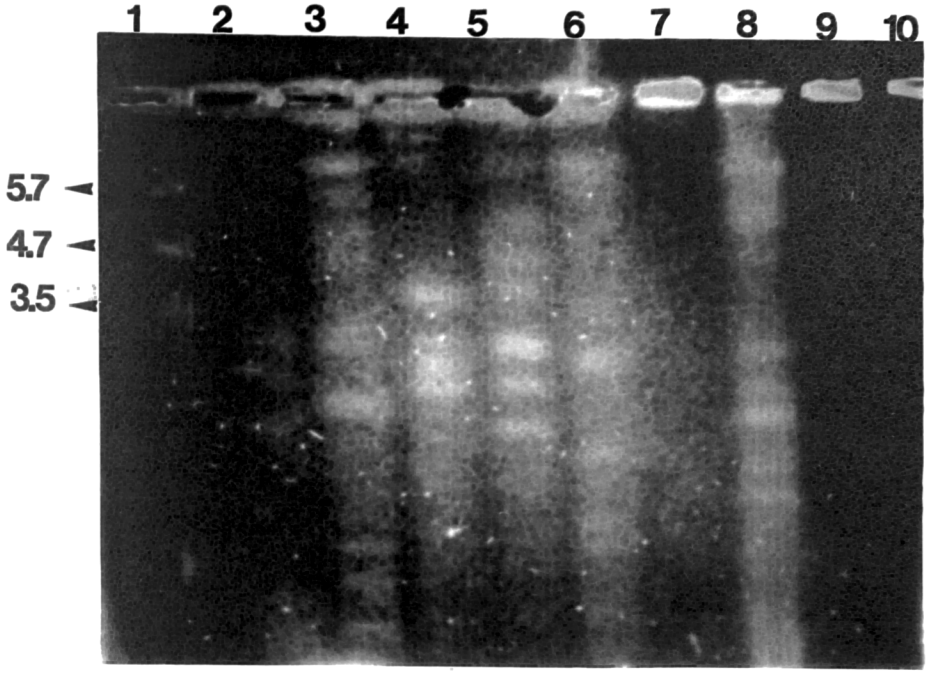


Plate-3.14-Contour-clamped, homogenous electric field pulsed field, gel electrophoresis (PFGE-CHEF) of chromosome-size DNA molecules. Electrophoretic karyotypes of representative isolates of *V.dahliae* (140, G17, lines: 2,4, respectively); *V.d.longisporum* (161, line 8) and putative recombinants of *V.d.longisporum* (111, G22, 195, lines: 3, 5, and 6 respectively) from the three groups (A, B and R) respectively. The run parameters were as follows: Switch time 3600 sec decreasing to 1000s during 264h. Field strength :50V. DNA size standards (5.7, 4.7, and 3.5) are given on the left in Mbs (*S.pombe*).

Table-3.19-Chromosome-length bands in isolate 161 of *V.d.longisporum* (Group B), isolates G17 and 140 of *V.dahliae* (Group A) and 3 *V.d.longisporum* recombinant isolates 111, 195, and G22 (Group R) resolved by PFGE-CHEF. Bands are in Mbs.

ISOLATES	140	111	G17	G22	195	161	G19	G859
BANDS	4	9	5-(6)	7	8-(10)	9-(10)	NR	NR
7.271	-	-	+	-	-	+	NR	NR
6.438*	-	+	+(?)	+	+ •	+ •	NR	NR
5.7	-	+	-	+	-	-	NR	NR
4.972**	-	+	-	-	+	+	NR	NR
4.337***	-	+	-	+	+	+	NR	NR
4.019	+	-	+	+	-	-	NR	NR
3.559					+		NR	NR
3.152	+	+	+	+	-	-	NR	NR
2.877	+	-	+	-	+	+	NR	NR
2.548	-	+	+	+	-	+	NR	NR
2.188	+	-	-	+	-	+	NR	NR
1.967	-	-	-	-	+	-	NR	NR
1.824	-	-	-	-	-	+	NR	NR
1.519	-	-	-	-	+	+	NR	NR
1.366	-	+	-	-	+	-	NR	NR
1.191	-	+	-	-	-	-	NR	NR
0.993	-	+	-	-	+(?)	-	NR	NR
Genome size	12.234	30.697	26.305	28.382	28.028	33.977		

+ Denotes presence of a chromosome DNA band.

- Denotes absence of a chromosome DNA band.

NR: Not resolved

* Chromosome-length DNA band common in all isolates under test , except 140.

** Chromosome-length DNA resolved only by diploids undre test

*** Chromosome-length DNA band common in *V.d.longisporum.* and *V.d.longisporum* recombinants only.

• Maybe a double

• Ref. to Fig. 3.14

3.1.6. Field observations on oilseed rape infected with *V.d.longisporum* in Germany (June 1991)

In order to observe the disease of oilseed rape (OSR) caused by the fungus *V.d.longisporum*, a 2 week visit was made to experimental fields in Northern Germany (Rostock) during 1991.

Experimental fields (four main sites).

1. Experimental station of the "Institut fur Phytomedizin der Universitat" in Rostock. These OSR fields had become naturally infected with *V.d.longisporum*. The first report of the disease was in 1983 when only few of the plants were infected. In 1984, 2% of the plants were diseased; by1989-1990 40% of the plants were diseased. The crop rotation had employed was Rape-Wheat-Barley-Rape since the year 1970.
2. Institut Fur Oil und Futter Phlanzenzuctung "Hanslembke" in Malcolch.. Breeding & selection for resistance to *V.d.longisporum*. The soils had been artificially infested with microsclerotia of the fungus in a Rape-Wheat-Barley-Rape rotation..
3. Breeding & selection for resistance in OSR to *V.d.longisporum* in Hohenlieth, near Kiel. Field soils had been artificially with residues of infected OSR and microsclerotia *of V.d.longisporum*. The rotation used here was 50% rape and 50% alternative crop so that the rape was grown every other year.
4. Experimental station in Fehmarn. Field soils had artificially infested soil with residues of *V.d.longisporum* infected OSR material.

The progress of the disease was observed to be very slow and it was usually difficult to identify *Verticillium*-infected plants by their external appearance before the ripening stage. This was because at the early stage of infection, the typical symptoms of the disease such as leaf chlorosis and leaf shedding can be caused by other fungi, as well as senescence.

In naturally and artificially infected fields symptoms of the disease started to appear just before "swath" (seed development stage: 6.7-6.8, when most seeds are black and hard). The diseased plants frequently showed a faint black shadow (Plate-3.17a- and-3.17b-) running up the stem; when the epidermis was peeled back (Plate-3.18-), black discolouration could be seen clearly in the outer cortical region. Striped or unilateral discoloration of the stem was a frequent and clear symptom of Verticillium-diseased plants at this stage (Plate-3.19a-, 3.19b). Infected stems often developed microsclerotia on the epidermis that germinate and sporulate giving a "powdery" external appearance to the plant (Plate -3.21-). The pith was also colonized and contained black microsclerotia (Plate-3.22-.).

Infected plants both ripened prematurely and showed yield reduction, these consequences being the two main problems associated with Verticillium wilt of OSR in the field. Frequently these effects were caused by a combination of *Verticillium* and *Phoma lingam* infection i.e. it can be a disease complex in the field (Plate-3.23-.).

Temperature is a very important factor for the development of symptoms because it affects the physiological maturity of the plant. Symptoms were observed to be delayed in OSR fields in Malchow/Poel compared with fields in Rostock due to the difference in temperatures (lower in the former).

The severity of the disease in the field was assessed on an arbitrary scale disease index every week, for 4 weeks (once a week) just before swath, as follows:

Score scale for field observations:

1.No symptoms

3.Stripped black discolorations of the stem (Plate-3.17a-, 3.17b-).

5.Yellow to brown discoloration of the whole stem. The same discoloration appeared in the vascular bands internally (Plate -3.19a-, -3.19b-).

7.Unilateral necrosis of the plant with dark microsclerotia appearing at one side of the plant (Plate -3.20-).

9. Microsclerotia developed around the whole stem (Plate-3.21-). Death of the plant.

Symptoms of Verticillium disease in OSR are highly related to maturity, even though the pathogen is able to infect and colonize the plant at an early stage of plant development as shown by artificial infection studies (Baig,1991; Zeise and Seidel 1990). For this reason, it was very difficult to infect and reproduce the symptoms in artificially infected seedlings that were grown under greenhouse conditions. Observations were made on the methods used to inoculate seedlings in Rostock. In order to obtain symptoms in the greenhouse consistently at Rostock, seedlings were artificially infected as follows: Two week-old plants were uprooted and the terminal ends of the root cut off to allow direct entry of the inoculum, washed with sterile distilled water and the root system immersed in two-week old blastospore suspension ($1X10^6$ blastospores/ml) for 2h and grown under stress conditions. They were grown in very small pots (5cm X5cm) at high plant density without fertilization and with continuous white cool light (500W HQL) at 22^0C-23^0C (Zeise,1992). Under these conditions, non-inoculated, control plants were developed yellow/orange brown leaves whereas the infected plants developed yellow/orange brown leaves with black coloured veins (Zeise and Seidel,1990), initial due to phenolic material and later by the production of microsclerotia. The following score system was used for the artificially infected OSR plants by Dr Karin Zeise in Rostock. Plants were scored three weeks after inoculation for four weeks.

Score scale for artificially infected seedlings growing under green-house conditions.

1. No symptoms
2. A few veins of older leaves discoloured (black).
3. Severe symptom development on oldest leaves.
4. Loss of leaves which first showed symptoms.
5. 50% of the leaves show severe symptoms
6. 50% of the leaves are lost.
7. More than 50% leaves are lost.
8. 100% of leaves lost.
9. Plant dead.

The same score scale was used for rapid cycle Brassicas that were infected with the fungus by the root-dipping method ($1X10^6$ blastospores/ml) and grown under greenhouse conditions. Observations were made three weeks after inoculation (just before ripening maturity) for four weeks.

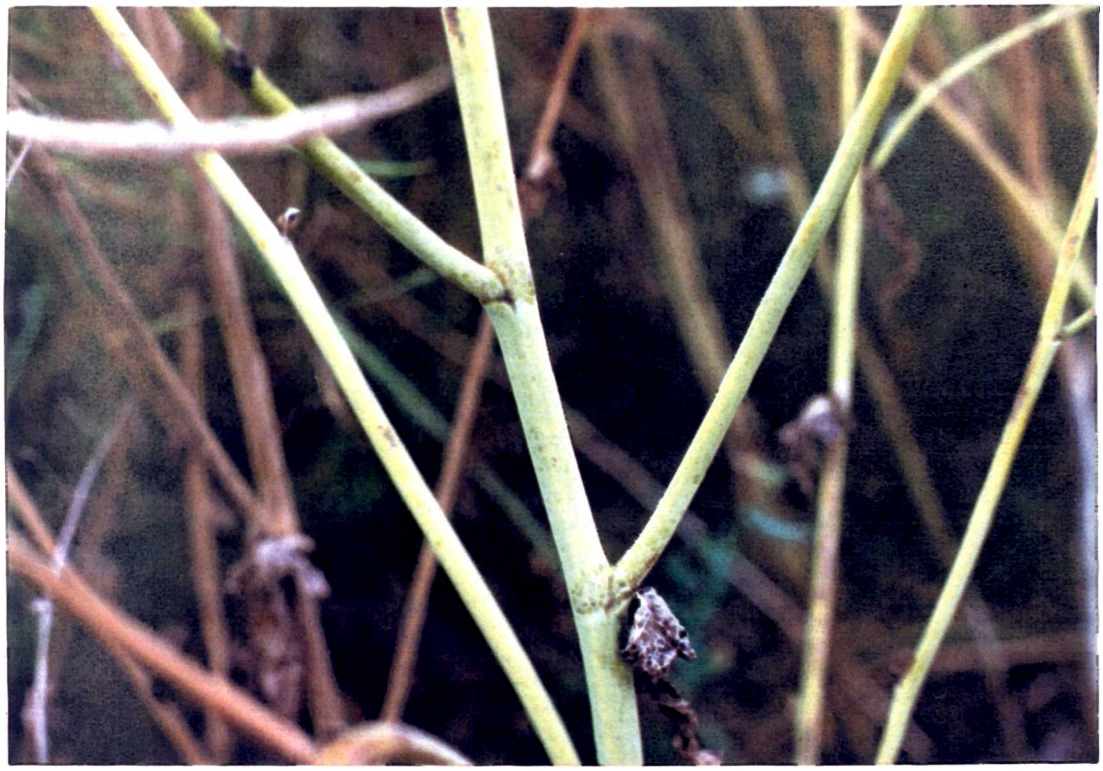


Plate-3.15-.Healthy plant of OSR in naturally infected field in Rostock, Germany. The stem has no marked discolouration or any sign of black microsclerotia of *V.d.longisporum*.

Plate-3.16-Healthy plant of OSR in Rostock, Germany. Peeling the epidermis of the stem of a healthy OSR plant showed no sign of black microsclerotia of *V.d.longisporum*.

Plate-3.17a- Apparently healthy plant of OSR in naturally infected field in Rostock, Germany. By careful examination a faint, darker strip ed discolouration can be just discerned, a possible indication of infection by *V.d.longisporum*.

Plate-3.17b- By rubbing the surface, a black discolouration (arrow-head) is seen at the surface of the outer cortex due to the presence of the fungus *V.d.longisporum* (disease score 3). Field infection Rostock, Germany.

Plate-3.18- By peeling back the epidermis and outer cortex of the above plant (Plate-3.17b-.), black discolouration can be seen (field infection, Rostock, Germany).

Plate-3.19a- Unilateral discolouration of an infected OSR plant caused by *V.d.longisporum* in Rostock, Germany.

Plate-3.19b-Unilateral discolouration of the intact stem of OSR caused by *V.d.longisporum*, as seen externally, disease score 5. (Rostock, Germany).

Plate-3.20- Development of black microsclerotia (arrow heads) of *V.d.longisporum* at a late stage within the epidermis and underlying cortical tissues of infected plant of OSR; disease score 7. (Rostock, Germany).

Plate-3.21- Microsclerotia of *V.d.longisporum* appear around the whole stem. The plant i.s necrotic/dead; disease score 9 (Rostock, Germany).

Plate-3.22- Pith of infected OSR plant is fully colonized by black microsclerotia of *V.d.longisporum* as seen in a vertical section of this plant (Rostock, Germany). [Isolates GV165, GV166, GV167, GV168, GV169,and GV170 used in this investigation, had been isolated from a similar *V.d.longisporum* infected-pith].

Plate-3.23- Plant of OSR plant doubly infected with *Phoma lingam* (pycnidia, arrow head) and *Verticillium dahliae var. longisporum* (microsclerotia, arrow). The infection by *Phoma lingam* makes the *Verticillium disease* even more severe (Rostock, Germany).

3.1.7.1. Verticillium pathogenicity tests using OSR seedlings under semi-controlled greenhouse conditions at King's College London.

In preliminary pot experiments in the greenhouse, attempts were made to inoculate 2-week-old seedlings of oilseed rape (OSR) growing in a soil mixture (John Innes/Levington's/sand mixture, 1:1:1) as reported by Baig (Baig,1991), but typical symptoms of the disease were not observed and the mixture employed also interfered with the subsequent extraction procedure for the enzyme myrosinase. For this reason, plants were subsequently grown in Vermiculite in high plant density (one seedling /4 cm pot) fed with half strength Phostrogen. Because the seedlings at first germinated in Vermiculite and after 2 weeks the vermiculite had to be removed before the root-dipping/inoculation procedure, some extra damage to the root was inevitable. No doubt this increased direct vascular infection and resulting in consistent symptom development. Also the use of small pots limited the root system, thus aiding the development of the disease.

Plants were inoculated by the root-dipping method with $1X10^6$ semi-synchronous spores/ml (section 2.3). An isolate was considered to be virulent when it produced symptoms and when the presence of the fungus in the vegetative parts was later confirmed by re-isolation and light microscopic examination. Eight haploid isolates in Group A of *V.dahliae* (table-3.20-), seventeen isolates in Group B viz. *V.d.longisporum* (table-3.21-) and the three putative recombinants of Group R (G22, G19,195, table-3.22-), were tested by inoculation against OSR cv. Cobra. To confirm the pathogenicity of isolates of the diploid strain: *V.d.longisporum*, four more winter OSR cvs, Envol, Idol, Samourai and Falcon (see table-2.2-) were tested against 3 isolates (G859, 161, G334) of the diploid strain viz. *V.d.longisporum* and one isolate (130) in Group A of an haploid (tomato) strain of *V.dahliae* which was expected to be avirulent to OSR.

Symptoms were assessed at 11 days, and subsequently at 15, 18, 20, 23, 25, 27 and 32 days after inoculation, using the key as described in section 2.3.6. All the virulent isolates produced marked stunting symptoms 15 days after inoculation, as compared with the avirulent isolates which had no significant effect on the growth of the plants. Infection reduced both the length of the petioles as well as the overall plant height (almost 100%). Chlorosis (yellowing) of the cotyledons of inoculated plants with virulent isolates started 11-15 days after inoculation, while non-inoculated control plants, and those of plants inoculated with avirulent isolates of the haploid strains of *V.dahliae* (table -3.20-), remained healthy and green even 32 days after inoculation. Defoliation was another symptom caused by virulent isolates of the fungus. Adult leaves of infected plants first showed chlorosis (yellowing) with some necrosis (browning) 15-18 days after inoculation (see Plate-3.26-). The symptoms developed first in the lower leaves and gradually moved upwards. When cut, infected stems and petioles exhibited vascular browning, being characteristic symptoms of Verticillium wilt. Control plants and plants infected with avirulent isolates grew without showing any visible symptoms, developing their 6th adult (true) leaf by the 32 day after inoculation. Death of the plants resulted with almost all virulent isolates of Group B (*V.d.longisporum*) after one month, post-inoculation. Plates -3.24,-3.25,-3.26.-, display the control uninoculated healthy OSR plants, the healthy plants inoculated with an haploid isolate of *V.dahliae* (130)

of Group A, and the diseased plants infected with a diploid isolate of *V.d.longisporum* (161)-of Group B respectively, 15 days after inoculation.

The mean disease score was calculated for all isolates/cultivars combination 11, 15, 18, 20, 23, 25, 27 and 32 days after inoculation. Also the disease integrate (Amelung & Schwiemann, 1986) was calculated using the formula:

$$Dj=\sum_{i+1}^{n}[(DS_{ti}+DS_{ti+1}/2)\ X\ (t_{i+1}-t_i)]$$

DS: disease score

ti: date of scoring

t_{i+1}: next date of scoring

$t_{i+1}-t_i$:difference in time

All 17 isolates of the diploid strain viz. *V.d.longisporum* were virulent to OSR cv.Cobra. Table-3.21- gives the disease integration for all isolates of Group B tested against cv. Cobra. All isolates of the haploid strains *V.dahliae* were avirulent to the same cv. Cobra. Table-3.20-. gives the disease integration for all haploid isolates of Group A tested against cv. (Cobra). Putative recombinants of *V.d.longisporum* (Group R) were slightly virulent or avirulent to cv. Cobra. Table-3.22- gives the disease integration for 3 putative recombinants tested against cv. Cobra (isolate 111 could not be tested because it formed insufficient spores).

Table-3.20- Host origin, disease integrate and pathogenicity of 8 isolates representing different haploid strains of *V.dahliae* (Group A) tested against OSR cv. Cobra by root-dip inoculation (section 2.3.).

ISOLATES	HOST	DISEASE INTEGRATE	PATHOGENICITY
130	Tomato	0	Avirulent
133	Tomato	0	Avirulent
140	Eggplant	0	Avirulent
362	Shepherd's purse	0	Avirulent
G1	Clover	0	Avirulent
G16	Potato	0	Avirulent
G17	Stock	0	Avirulent
GR1	Olive-tree	0	Avirulent

Table-3.21- Host origin, disease integrate and pathogenicity of 17 isolates of the diploid strain: *V.d.longisporum* (Group B) tested against OSR cv. Cobra by root-dip inoculation (section 2.3.).

ISOLATE	HOST	DISEASE INTEGRATE	PATHOGENICITY
161	OSR	71.104	Virulent
162	Sugar beet	27.395	Virulent
86207	Chinese radish	44.958	Virulent
84013	Chinese cabbage	56.791	Virulent
84120	Chinese cabbage	66.791	Virulent
G10	OSR	70.437	Virulent
G18	OSR	50.414	Virulent
G25	OSR	45.020	Virulent
G23	OSR	67.718	Virulent
G29	OSR	46.541	Virulent
G34	OSR	33.875	Virulent
GV167	OSR	47.770	Virulent
F1	OSR	65.156	Virulent
G334	OSR	73.083	Virulent
F617-7	OSR	81.208	Virulent
F654-2	OSR	43.770	Virulent
S856*	OSR	72.750	Virulent

* S856=G856

Table-3.22- Host origin, disease integrate and pathogenicity of 4 putative recombinant strains, probably derived from the diploid strain: *V.d.longisporum* (111, G22, 195, G19) tested against OSR cv. Cobra by root-dip inoculation (section 2.3.).

ISOLATE	HOST	DISEASE INTEGRATE	PATHOGENICITY
111 [1]	Brussels sprout	-	Slightly virulent [2]
G22	OSR	0	Avirulent
195	Horse radish	6.375	Slightly virulent
G19	OSR	7.479	Slightly virulent

[1] This isolate could not be tested by the author because it formed insufficient spores.

[2] This isolate111 was tested by Baig,1991 and found to be slightly virulent to OSR.

To test whether there was heterogeneity among the mean disease score for all 17 isolates of the diploid strain *V.d.longisporum* tested against cv. Cobra the single factor Anova was performed. For all diploid isolates (Table-3.21-) tested against cv. Cobra, the found values for F were higher than the tabulated F values. There was a highly significant difference (p=0.001) among the mean disease score for all 17 diploid isolates of *V.d.longisporum* tested against OSR cv. Cobra (Table-3.23-). So all isolates tested against cv. Cobra did not came from the same population.

Table-3.23- Analysis of variance (Single Factor Anova) of disease score-data of 17 isolates of the diploid strain viz. *V.d.longisporum* (Group B) tested against seedlings of OSR cv. Cobra.

DAYS POST INOCULATION	F VALUE	TABULATED F VALUE
11	9.376*	2.533 at p=0.001
15	15.518*	2.533 at p=0.001
18	28.201*	2.533 at p=0.001
20	23.877*	2.533 at p=0.001
23	24.087*	2.533 at p=0.001
25	15.259*	2.533 at p=0.001
27	12.263*	2.533 at p=0.001
32	9.513*	2.533 at p=0.001

*Denotes F-values highly significant at p=0.001 level.

The correlation between disease integrate and mean disease for all 17 virulent isolates tested against cv. Cobra at 23 days post-inoculation was very highly significant even beyond the 0.001 probability level (r=0.986, see following figure-3.9a-).

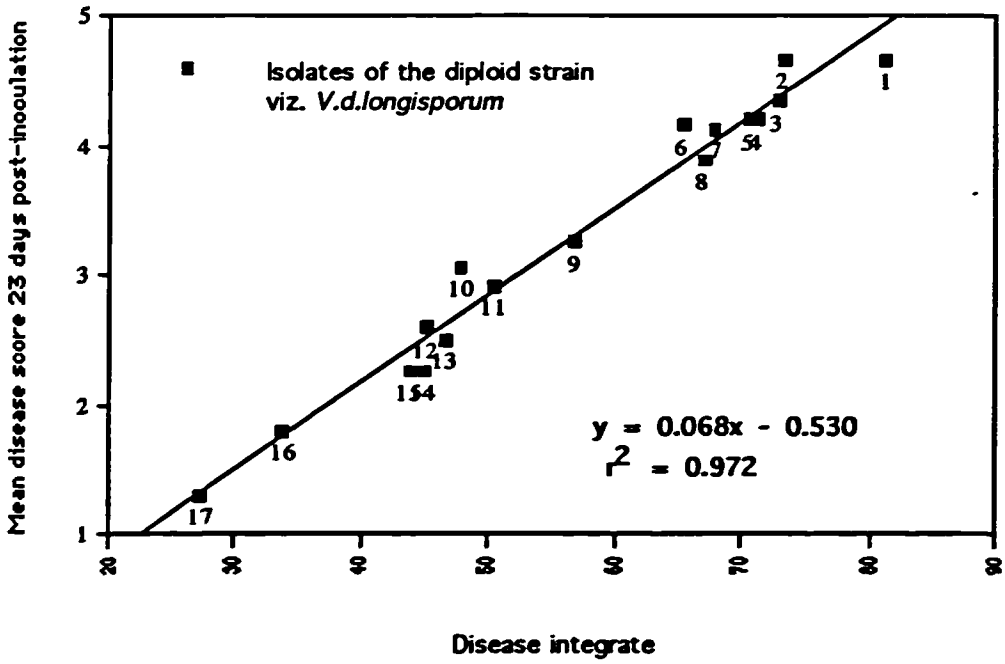


Figure-3.9a- Correlation between disease integrates and mean disease scores (at 23 days post inoculation) in OSR cv. Cobra. (r=0.096, highly significant at p< 0.001). For isolate descriptions see Table-3.21-.

To test which particular isolates were significantly different from which others, the least significant difference was found (LSD) for the mean disease scores at 23 days after inoculation (see table 3.23 above). There was no significant difference between the virulence of the first isolate which showed the highest mean disease score and the other six isolates (F617-7[a], G334 [a], S856 [ab], 161 [ab], G10 [ab], F1 [ab], and G23 [ab]). The same letter on the side of each isolate indicates that there was no significant difference between the two isolates . Also, there was no significant difference between the virulence of the isolates with the lowest mean disease score (G34, 162). The other isolates were of intermediate virulence. The mean disease scores for the two isolates 84120 [bc], 84013 [c] were not significantly different from each other but were significantly different from isolates with a lower mean disease score than the isolate F617-7 [a]. The next 4 isolates GV169 [cd], G18 [cd], G25 [de] and G29 [de] were of the same virulence, but were significanly different from isolates with lowest and higher mean disease scores. The two isolates 86207 [ef] and F654-2 [ef] were of the same virulence, but were significantly different from isolates with lowest and highest mean disease scores. Table-3.24- gives the result for the supplementary t-test for the mean disease score of all diploid isolates of *V.d.longisporum* tested against cv. Cobra 23 days after inoculation (residual degrees of freedom=391) .

Table -3.24- Statistical comparison employing a t-test for mean disease scores 23 days after inoculation (data for 17 isolates of the diploid strain viz. *V.d.longisporum* tested against OSR cv.Cobra).

NUMBER	ISOLATE	MEAN DISEASE SCORE 23 days post inoculation	DISEASE INTEGRATE	VIRULENCE
1	F617-7 [a]	4.666	81.2	Highly virulent
2	G334 [a]	4.666	73.1	
3	S856 [ab]	4.333	72.8	
4	161 [ab]	4.208	71.1	
5	G10 [ab]	4.208	70.4	
6	F1 [ab]	4.166	65.2	
7	G23 [ab]	4.125	67.8	
8	84120 [bc]	3.875	66.8	Very Virulent
9	84013 [c]	3.250	56.8	
10	GV169 [cd]	3.041	47.8	Virulent
11	G18 [cd]	2.914	50.4	
12	G25 [de]	2.583	45	
13	G29 [de]	2.500	46.5	
14	86207 [ef]	2.250	45	Less virulent
15	F654-2 [ef]	2.250	43.8	
16	G34 [fg]	1.791	33.9	Mildly virulent
17	162 [g]	1.291	27.4	
	LSD=0.642 at P=0.05			

The development of symptoms in inoculated seedlings of OSR cv. Cobra employing the 17 isolates of the pathogenic diploid strain viz. *V.d.longisporum* are given in figures-3.10, 3.11 ,3.12 ,3.13, 3.14 and 3.15. Figure-3.16 - gives the comparative result for mean disease scores for all isolates of the three groups tested against OSR cv. Cobra 23 days after inoculation.

All three isolates (161, G334 and G859) of the diploid strain viz. *V.d.longisporum* were virulent to the 4 other OSR cvs tested. Isolate 130 of the haploid strain *V.dahliae* was avirulent to these same cultivars. Table-3.25- gives the disease integration for all 4 cvs and 4 isolates combination tested.

Table-3.25- Host origin, disease integrate and pathogenicity of one isolate (130) of an haploid strain of *V.dahliae* (Group A) and 3 isolates of the diploid strain viz. *V.d.longisporum* (Group B) tested against 4 OSR cvs: Envol, Idol, Samourai and Falcon by root-dip inoculation (section 2.3.).

ISOLATE	HOST	DISEASE INTEGRATE ENVOL	DISEASE INTEGRATE IDOL	DISEASE INTEGRATE SAMOURAI	DISEASE INTEGRATE FALCON	PATHOGENICITY
130	Tomato	0	0	0	0	Avirulent
161	Sugar beet	55.9	59.4	47.4	63.4	Virulent
G334	OSR	52.7	58.5	37.8	59.4	Virulent
G859	OSR	48	51.3	32.5	54.4	Virulent

The correlation between mean disease scores 23 days after inoculation and mean disease integrates in the 4 OSR (Falcon, Idol, Envol and Samourai) produced by the 3 virulent isolates 161, G334 and G859 was very highly significant even beyond the 0.001 probability level (r=0.948 see figure below).

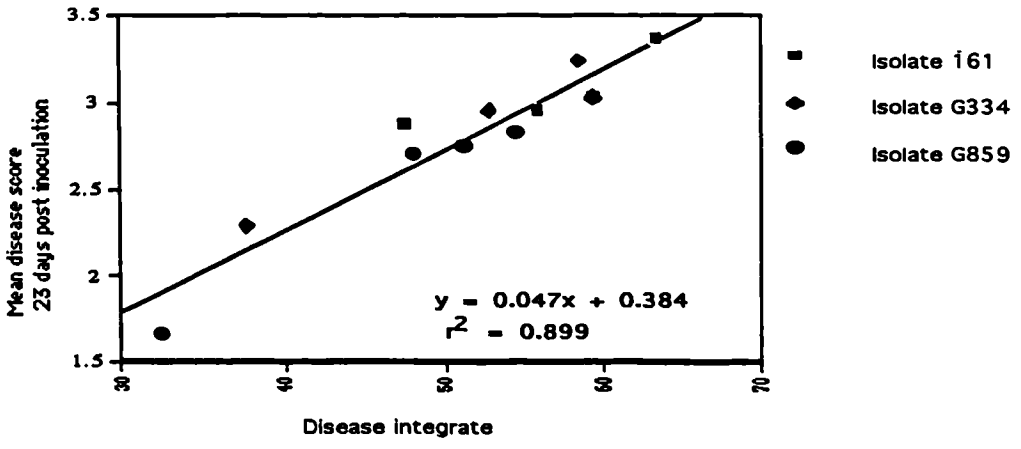


Figure-3.9b- Correlation between disease integrates and mean disease scores (23 days post inoculation) in 4 OSR cvs (Falcon, Idol, Envol and Samourai) (r=0.948, highly significant at $p < 0.001$). For isolate descriptions see Table-3.21

To test whether the 3 isolates of the diploid strain viz.*V.d.longisporum* tested against the 4 OSR cvs. were of the same virulence, the single factor Anova was performed. For all diploid isolates (Table-3.25-) tested against cv. Envol, the found value for F was lower than the tabulated F value (p=0.05) 23 days after inoculation. There was no significant difference (p=0.05) among the virulence of all 3 diploid isolates (161, G334, G859) of *V.d.longisporum* tested against OSR cv. Envol 23 days after inoculation (Table-3.26-).

For all diploid isolates (Table-3.25-) tested against cv.Idol, the found value for F was lower than the tabulated F value (p=0.05) 23 days after inoculation. There was no significant difference (p=0.05) among the virulence of all 3 diploid isolates of *V.d.longisporum* tested against OSR cv. Idol, 23 days after inoculation(Table-3.26-).

For all diploid isolates (Table-3.25-) tested against cv.Samourai, the found value for F was higher than the tabulated F values (p=0.05) 23 days post inoculation. There was a highly significant difference (p=0.01) between the virulence of all 3 diploid isolates of *V.d.longisporum* tested against OSR cv. Samourai 20 and 23 days after inoculation(Table-3.26-). The LSD was found for the mean disease score 23 days after inoculation at p=0.05. Isolates161 and G334 were not significantly different but were significant more virulent than G859 (see table-3.27-).

For all diploid isolates (Table-3.25-) tested against cv.Falcon the found value for F was lower than the tabulated F (p=0.05) values 23 days post inoculation. There was no significant difference (p=0.01) among the virulence of all 3 diploid isolates of *V.d.longisporum* tested against OSR cv. Falcon 23 days after inoculation(Table-3.27-).

Table-3.26- Single factor anova (analysis of variance) for disease score data 23 days post inoculation for three isolates of *V.d.longisporum* tested against cvs: Envol, Idol, Samourai and Falcon.

DAYS POST INOCULATION	F-VALUE ENVOL	F-VALUE IDOL	F-VALUE SAMOURAI	F-VALUE FALCON
23	2.044	2.614	6.957*	2.814

*Denotes highly significant differences at p=0.01 (Tabulated value F=4.98 at p=0.05).

Table-3.27- Statistical differences in disease scores employing a t-test for three diploid isolates of the pathogenic strain viz. *V.d.longisporum* tested against the 4 cvs. of OSR: Envol, Idol, Samourai and Falcon at 23 days post-inoculation.

ISOLATE	MEAN DISEASE SCORE	CULTIVAR
161	2.958 [a]	ENVOL
G334	2.958 [a]	ENVOL
G859	2.708 [a]	ENVOL
161	3.041 [a]	IDOL
G334	3.25 [a]	IDOL
G859	2.75 [a]	IDOL
161	2.875 [a]	SAMOURAI
G334	2.291 [a]	SAMOURAI
G859	1.666 [b]	SAMOURAI
LSD=0.709		
161	3.375 [a]	FALCON
G334	3.041 [a]	FALCON
G859	2.833 [a]	FALCON

Figures 3.17, 3.18, 3.19 and 3.20 show the development of symptoms in inoculated OSR cvs Envol, Idol, Samourai and Falcon respectively, using the virulent diploid isolates of Group B, viz. 161, G334 and G859. Figure-3.21- gives the comparative result for all isolates tested against cvs Envol, Idol, Samourai and Falcon respectively, 23 days after inoculation.

To test wheter there were any significant differences among the susceptibility/resitance of the 5 OSR cultivars (Cobra, Envol, Idol, Samourai and Falcon) for the two most virulent isolates 161 and G334 of the diploid strain viz. *V.d.longisporum* the two factor analysis of variance was employed for the mean disease score data 11, 15, 18, 20, 23, 25, 27 and 32 days post-inoculation . The calculated F values at 5% were higher than the tabulated ones indicating significant difference in susceptibility of the 5 cultivars to both isolates 161 and G334 (see Table-3.28-) for all the days post-inoculation

Table-3.28- Analysis of variance for mean disease scores of the 5 OSR cvs tested against two virulent isolates of the diploid strain viz. *V.d.longisporum* 161 and G334, at 11 to 32 days post-inoculation.

SOURCE OF VARIATION	F	F TABULATED p=0.05	DAYS POST INOCULATION
Isolate	1.280	3.882	11
Cultivar	6.781*	2.410	11
Interaction	0.648	2.410	11
Isolate	1.377	3.882	15
Cultivar	12.392*	2.410	15
Interaction	0.155	2.410	15
Isolate	1.868	3.882	18
Cultivar	28.638*	2.410	18
Interaction	5.263*	2.410	18
Isolate	1.037	3.882	20
Cultivar	34.560*	2.410	20
Interaction	5.805*	2.410	20
Isolate	0.311	3.882	23
Cultivar	48.581*	2.410	23
Interaction	4.310*	2.410	23
Isolate	10.588*	3.882	25
Cultivar	53.956*	2.410	25
Interaction	2.528*	2.410	25
Isolate	7.202*	3.882	27
Cultivar	42.226*	2.410	27
Interaction	0.260	2.410	27
Isolate	1.16	3.882	32
Cultivar	26.34*	2.410	32
Interaction	3.68*	2.410	32

* Denotes statistical difference at p=0.05

To test which cultivar was most susceptible to isolate 161, the single factor analysis of variance was performed for 23 days post-inoculation and the LCD was calculated at p=0.05. Cobra was the most susceptible OSR cv. of all the 5 cultivars, with significant differences in susceptibility from all the others. Table-3.29- presents the statistical comparison of all 5 cvs tested with the virulent isolate 161 of *V.dahliae longisporum.*

Table-3.29- Statistical comparison of susceptibility/resistance of 5 different OSR cvs tested with virulent isolate *V.d.longisporum*, 23 days after inoculation.

CULTIVAR	MEAN DISEASE SCORE
Cobra [a]	4.208
Falcon [b]	3.375
Idol [c]	3.041
Envol [c]	2.958
Samourai [c]	2.875
LSD=0.248 at p=0.05	

Two isolates, one virulent (161) of the pathogenic diploid strain viz. *V.d.longisporum* and one avirulent isolate (130) of a non-pathogenic, haploid strain of *V.dahliae* were tested against another host viz. Chinese cabbage. Inoculation with these two isolates gave similar results as with OSR cvs. (Plate-3.33-) although complete host death did not occur here, even 2 months after inoculation (Plate-3.34).

Isolations from the stem and petioles of the plants showing symptoms always yielded the fungus i.e. *V.d.longisporum* (Plate-3.35-) with the characteristics of the isolate used to inoculate the plants.

Plate-3.24- Symptomless, healthy control plants of OSR (cv. Cobra) 15 days after root-dipping in sterile distilled water only. Plants were grown under semi-controlled environment greenhouse conditions at King's College London (section 2.3.).

Plate-3.25- Healthy OSR plants (cv. Cobra), inoculated with an haploid isolate (130) of *V.dahliae* (tomato), 15 days after inoculation .Plants were grown in semi-controlled greenhouse conditions at King's College London and inoculated by root-dipping in a spore suspension of 1X 10^6 conidia/ml (section 2.3.).

Plate-3.26- Diseased OSR plants(cv. Cobra) infected with an isolate (161) of *V.d.longisporum* (sugar beet) with chlorotic and necrotic cotyledons and leaves, 15 days after inoculation. Plants were grown in semi-controlled greenhouse conditions at King's College London and infected by root-dipping in a spore suspension of 1X 10^6 conidia/ml (section 2.3.).

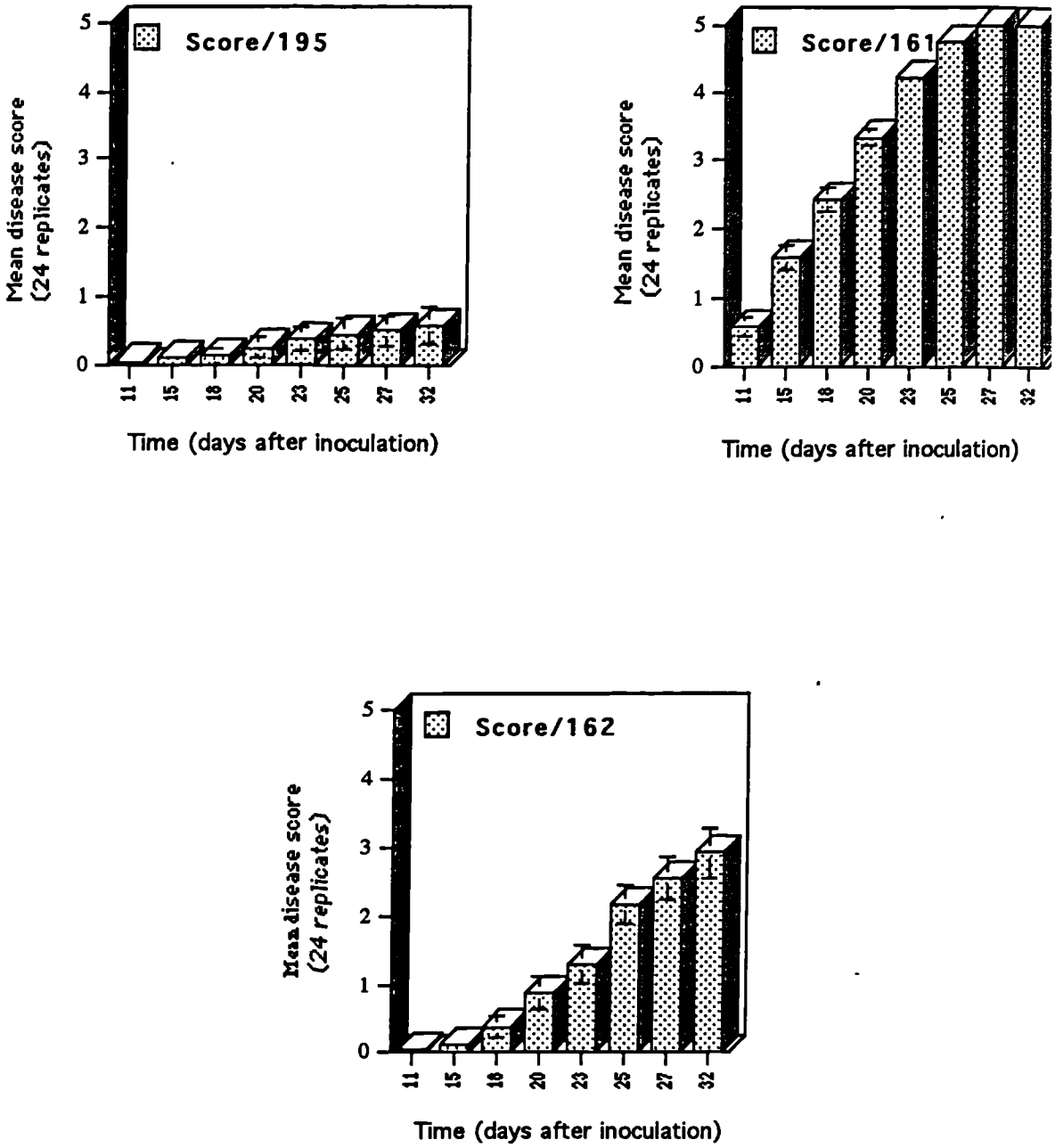


Figure -3.10- Development of symptoms in inoculated OSR cv. Cobra employing isolates 195(slightly virulent), 161(virulent) and 162(virulent) of the pathogenic diploid strain viz. *V.d.longisporum*. (Symptom score, methodology section 2.3). Vertical bars=standard error of the mean.

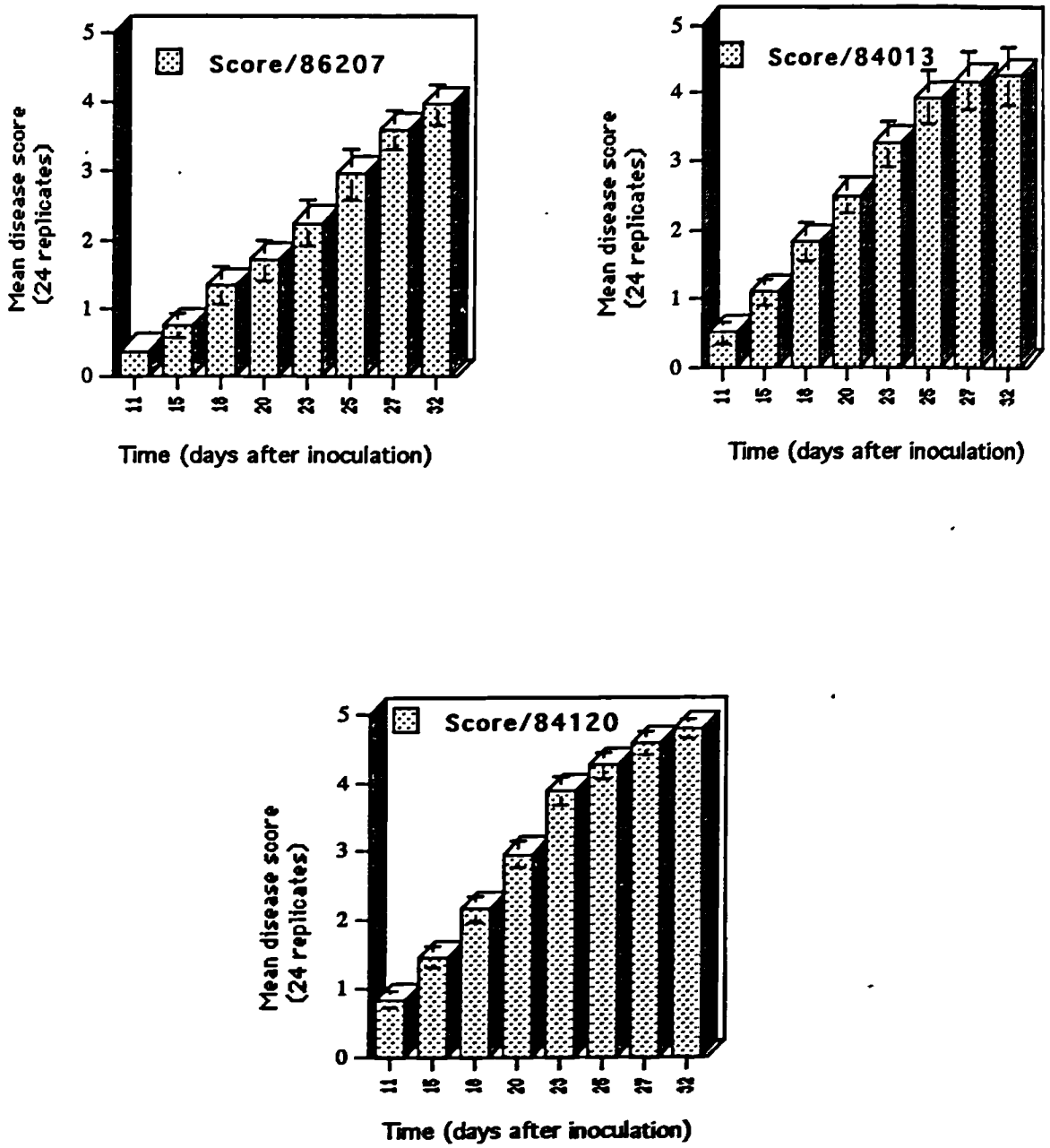


Figure -3.11- Development of symptoms in inoculated OSR cv. Cobra employing the virulent Japanese isolates 86207, 84120 and 84013 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score, methodology section 2.3). Vertical bars=standard error of the mean.

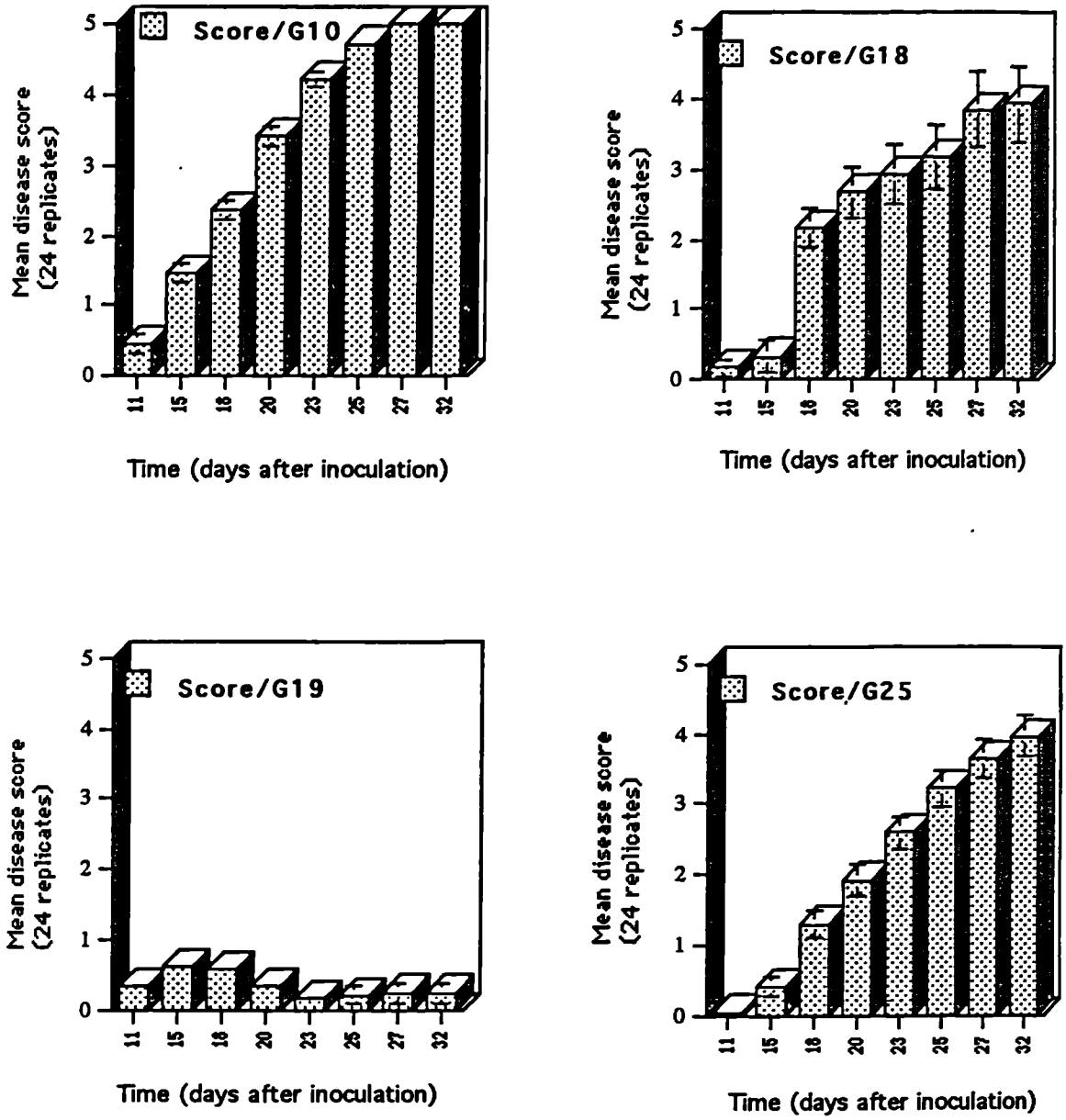


Figure -3.12- Development of symptoms in inoculated OSR cv. Cobra employing the slightly virulent (putative recombinant) isolate G19 and the virulent isolates G10, G18, and G25 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score, methodology section 2.3). Vertical bars=standard error of the mean.

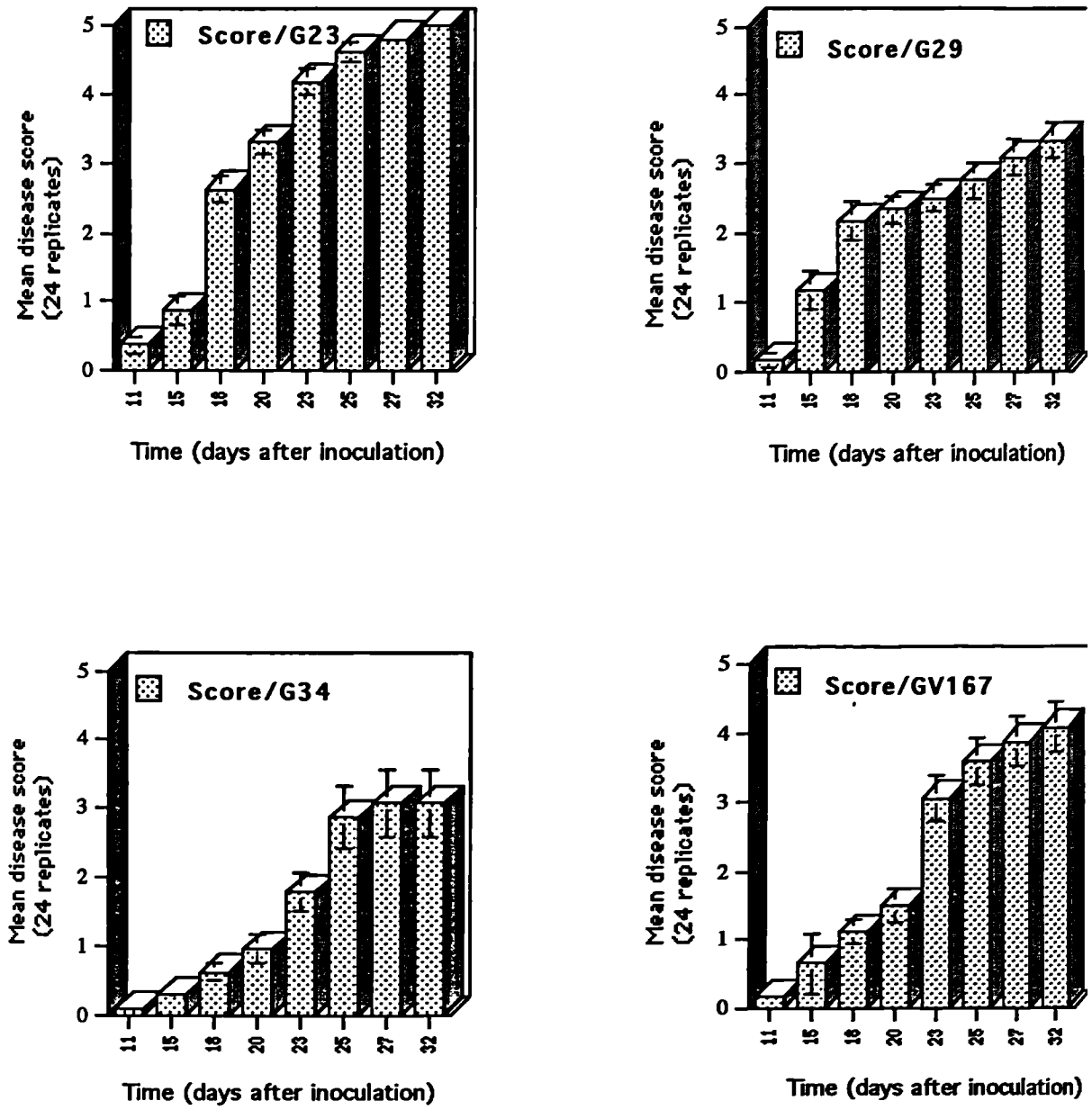


Figure -3.13-.Development of symptoms in inoculated OSR cv. Cobra employing the slightly virulent (putative recombinant) isolate G19 and the virulent isolates G10, G18, and G25 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score, methodology section 2.3). Vertical bars=standard error of the mean.

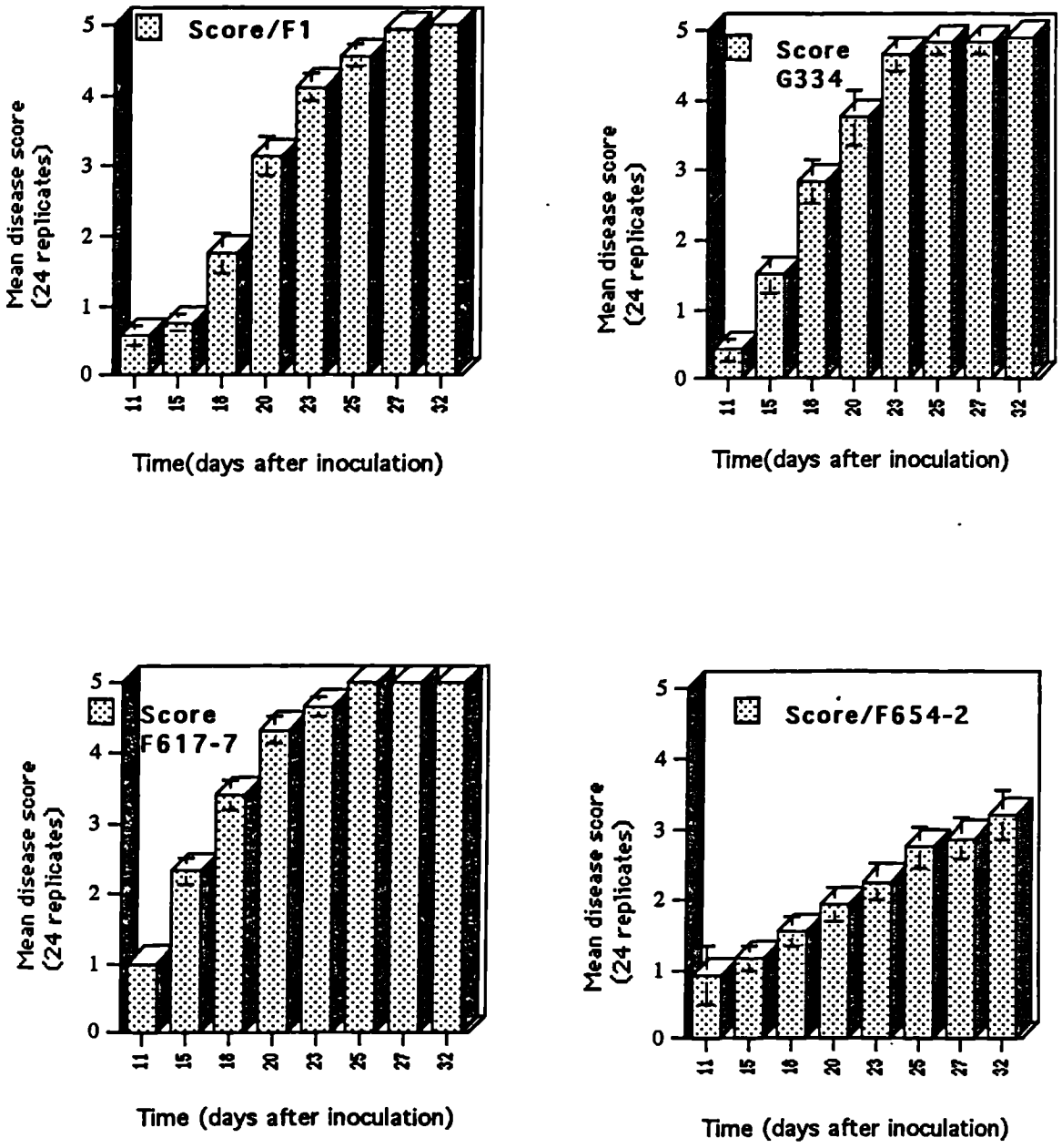


Figure-3.14- Development of symptoms in inoculated OSR cv. Cobra employing the virulent isolates F1, G334, F617-7 and F654-2 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score, methodology section 2.3). Vertical bars=standard error of the mean.

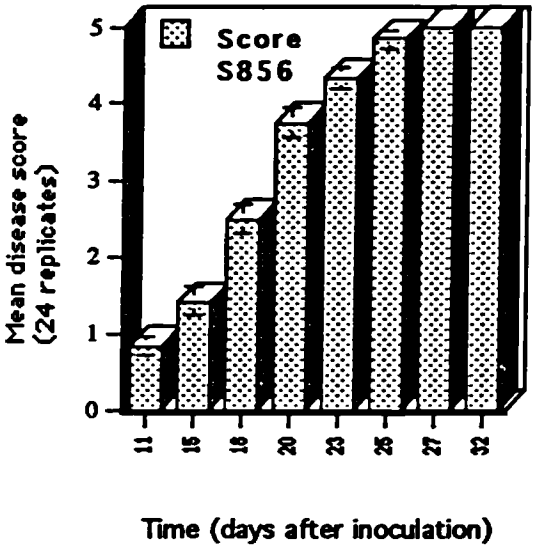


Figure-3.15-Development of symptoms in inoculated OSR cv. Cobra employing the virulent isolate S856 (=G856, Senickerande) of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score, methodology section 2.3). Vertical bars=standard error of the mean.

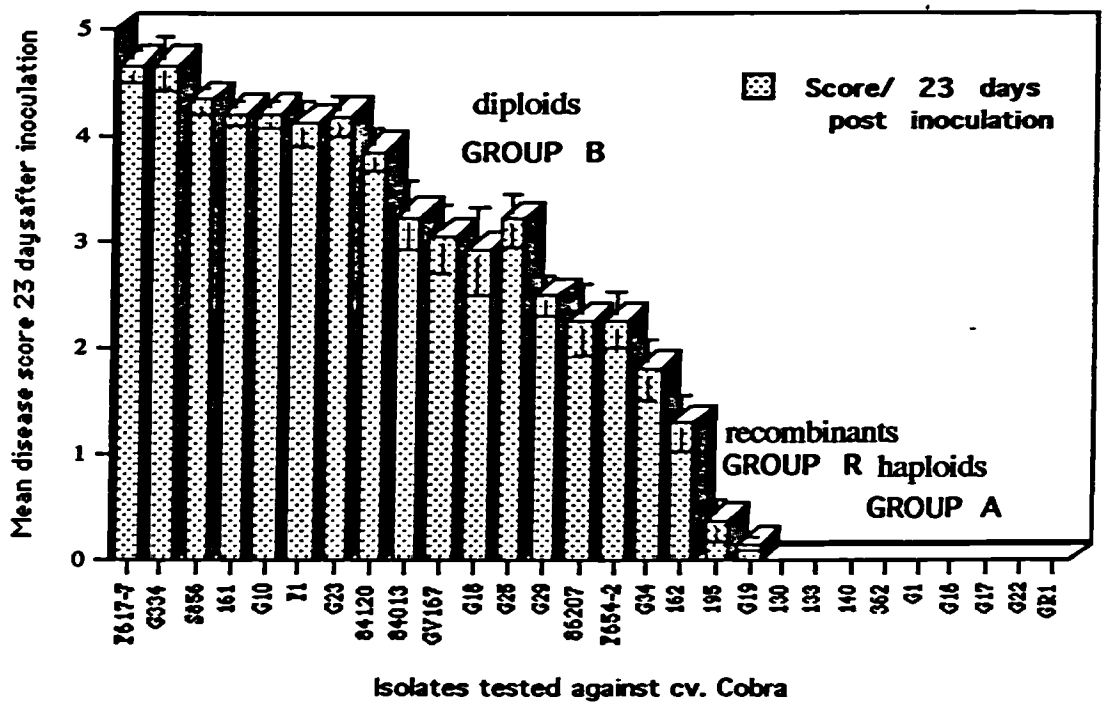


Figure-3.16-Mean disease scores of all isolates tested against OSR cv. Cobra 23 days after inoculation. Isolates 161-S856 are in Group B of the diploid pathogenic strain viz.*V.d.longisporum*; isolates 195, G19, G22 are in Group R i.e. the putative recombinant srtains of *V.d.longisporum*; isolates 130-GR1 are in Group A of the haploid strains of *V.dahliae*.

Table-3.29b-Lay-out of seedling pathogenicity tray for OSR cvs: Envol, Idol, Samourai and Falcon. Seedlings were inoculated with 3 virulent isolates(161, G859 and G334) of the pathogenic diploid strain viz. *V.d.longisporum* and one avirulent isolate 130 of the haploid strain *V.dahliae* by the root-dip method as described in section 2.3.4.2 and transferred to the tray according to the following lay-out. This test was performed in 3 replicate trays for each cultivar of OSR.

130	G334	CONTROL	161	G334	161	G859	CONTROL
G859	161	G859	G334	130	CONTROL	161	G334
G334	130	CONTROL	CONTROL	G859	130	130	G859
130	G859	CONTROL	161	G859	G334	CONTROL	130
161	CONTROL	161	G334	G859	161	G334	130

Plate-3.27-Seedling pathogenicity tray of OSR cv. Envol 20 days after inoculation. 2 weeks-old seedlings were inoculated by root-dipping and arranged in the greenhouse in seed pot /trays in a completely random block array according to the table-3.30-(as shown above) at King's College London.

Plate-3.28- Symptomless, control healthy plant of OSR cv. Envol 20 days after dipping in sdw, only with green cotyledons and 4 true leaves (King's College London).

Plate-3.29-Diseased plant of OSR cv. Envol (inoculated with isolate 161, diploid strain *V.d.longisporum*) showing symptoms of chlorosis. Both cotyledons are yellow as are two of the four true leaves (score 3), 20 days after inoculation (King's College London).

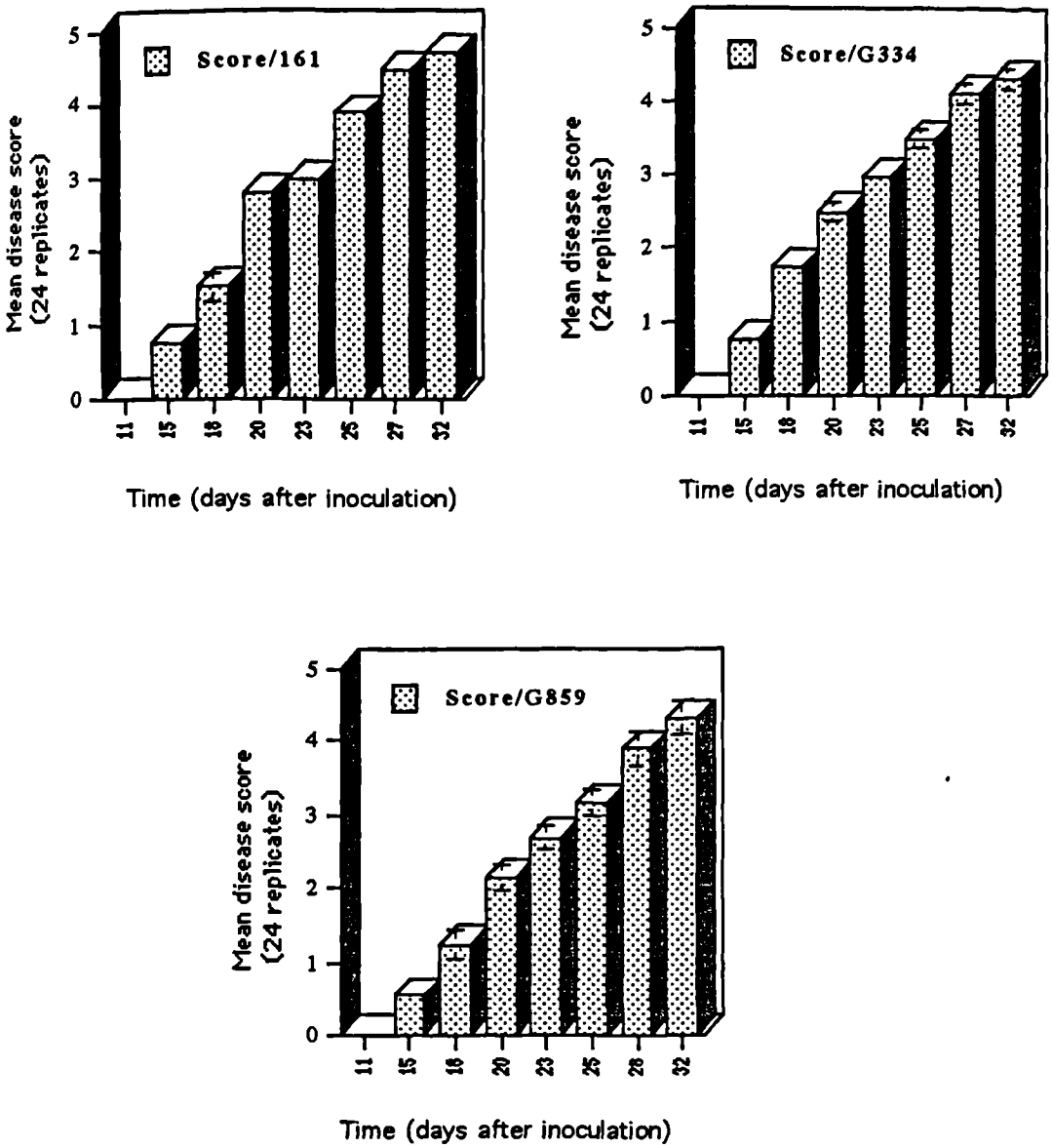
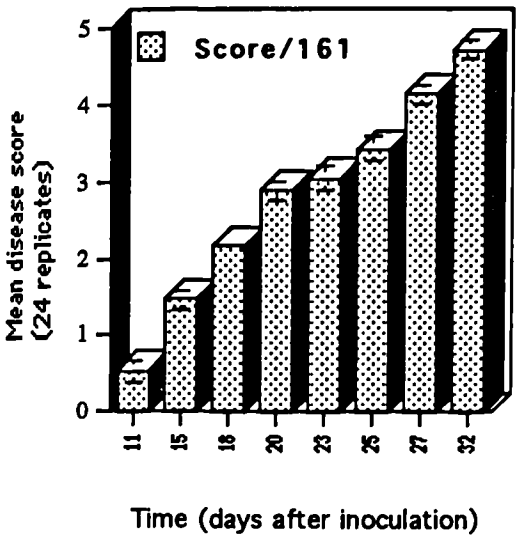
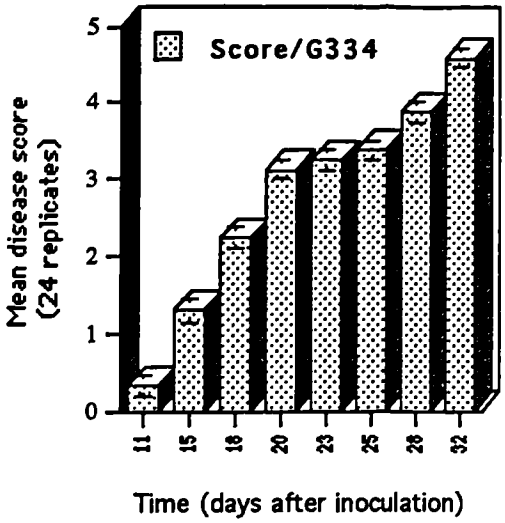


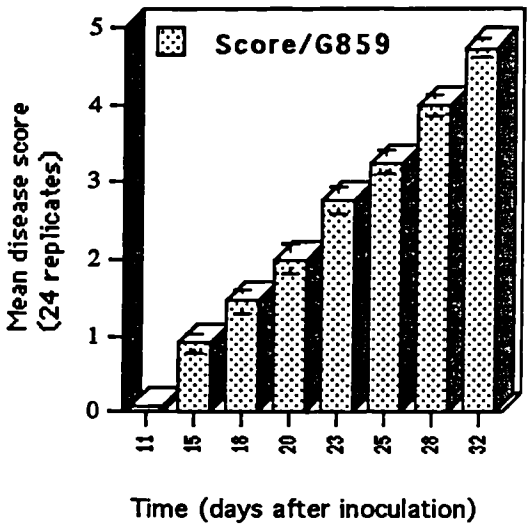
Figure-3.17- Development of symptoms in inoculated OSR cv. Envol employing the virulent isolates 161, G334 and G859 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score; methodology section 2.3.). Vertical bars=standard error of the mean.

Plate.-3.30- Seedling pathogenicity tray of OSR cv. Idol 15 days after inoculation. 2 week-old seedlings were inoculated by root-dipping and arranged in the greenhouse in seed pot /trays in a random block array according to the table-3.29b-(King's College London).

Plate-3.31- Seedling pathogenicity tray of OSR cv. Samourai 8 days after inoculation. 2 week-old seedlings were inoculated by root-dipping and arranged in the greenhouse in seed pot /trays in a random block array according to the table-3.29b (King's College London). Chlorotic and stunting symptoms have not started at this time.







igure-3.18-Development of symptoms in inoculated OSR cv. Idol employing the virulent isolates 161, G334 and G859 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score; methodology section 2.3.). Vertical bars=standard error of the mean.

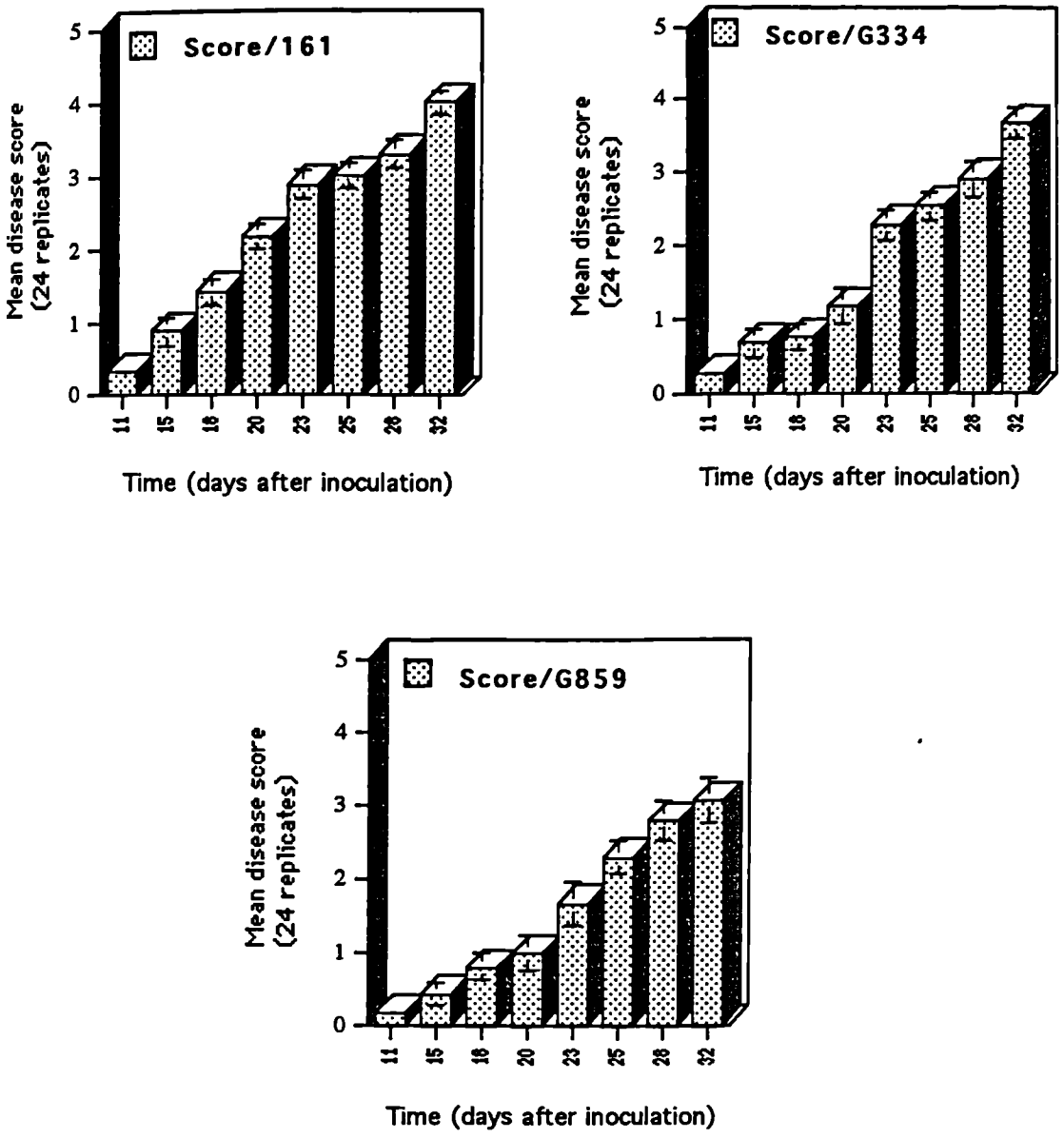


Figure-3.19- Development of symptoms in inoculated OSR cv. Samourai employing the virulent isolates 161, G334 and G856 of the pathogenic diploid strain viz.*V.d.longisporum*. (Symptom score, methodology section 2.3.). Vertical bars=standard error of the mean.

Plate-3.32- Seedling pathogenicity tray of OSR cv. Falcon 20 days after inoculation. Diseased plants showing yellow/chlorotic leaves and cotyledons. 2 week-old seedlings were inoculated by root-dipping and arranged in the greenhouse in seed pot /trays in a random block array according to the table-3.29b (King's College London).

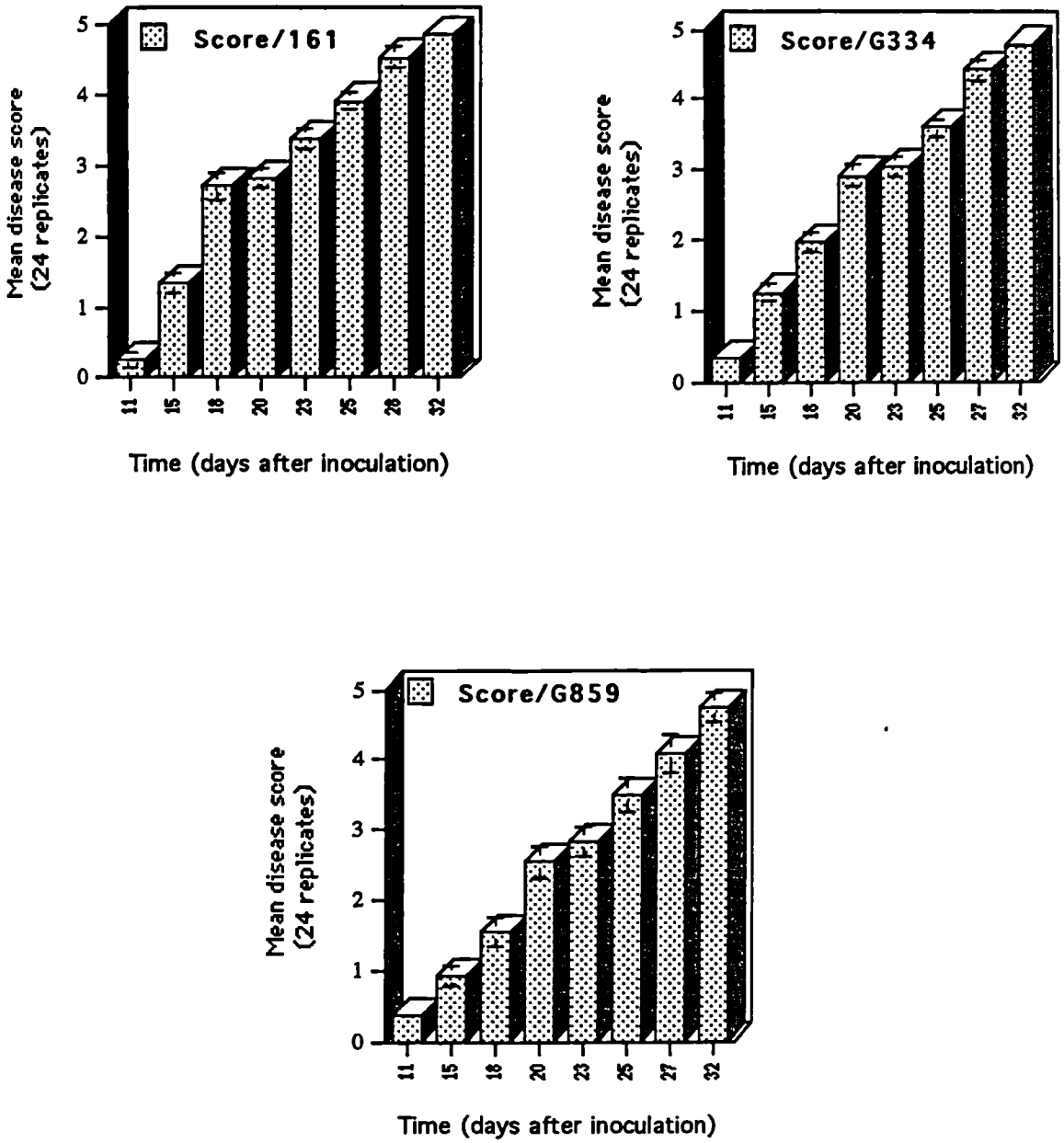


Figure-3.20- Development of symptoms in inoculated OSR cv. Falcon employing the virulent isolates 161, G334 and G859 of the pathogenic diploid strain viz. *V.d.longisporum*. (Symptom score, methodology section 2.3.). Vertical bars=standard error of the mean.

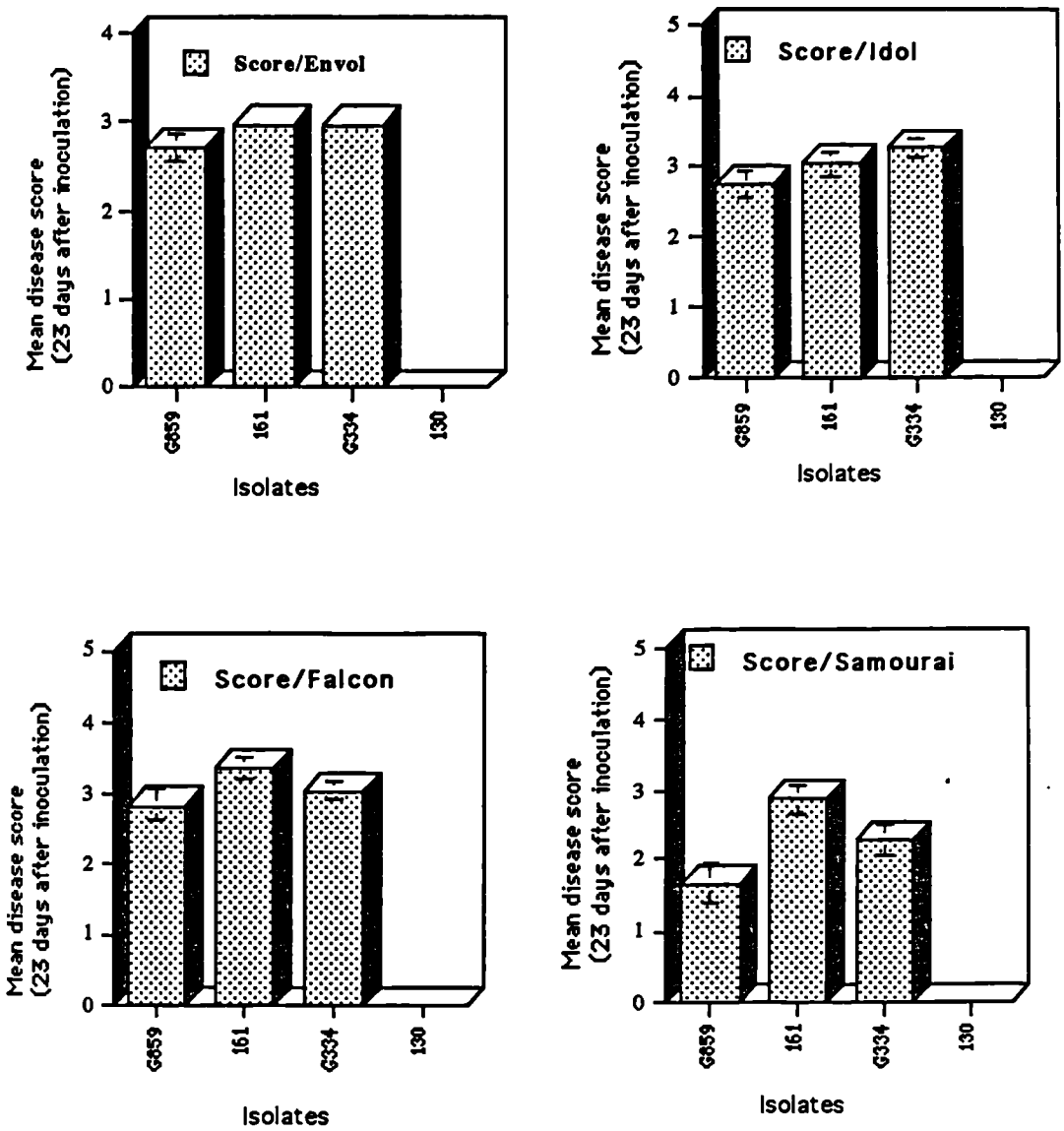


Figure-3.21-.Mean disease score of three isolates G859, 161 and G334 of the pathogenic diploid strain viz.*V.d.longisporum* and one avirulent isolate 130 of the non-pathogenic haploid strain of *V.dahliae* tested against four cultivars of OSR (Envol, Idol, Samourai and Falcon), 23 days after inoculation. There was no significant difference in virulence among the three virulent isolates (G334,161 and G859) of the diploid strain viz. *V.d.longisporum* tested against cvs Envol, Idol and Falcon 23 days after inoculation (p=0.05). There was a highly significant difference (p=0.01) among the virulence of the three virulent isolates (G334, 161 and G859) of the diploid strain viz. *V.d.longisporum* tested against cv. Samourai. Isolates 161 and G334 were significantly more virulent than G859 at p=0.05 (LSD=0.709). Isolate 130 of the haploid strain *V.dahliae* (tomato) was avirulent to all 4 cvs of OSR (Envol, Idol, Samourai and Falcon).

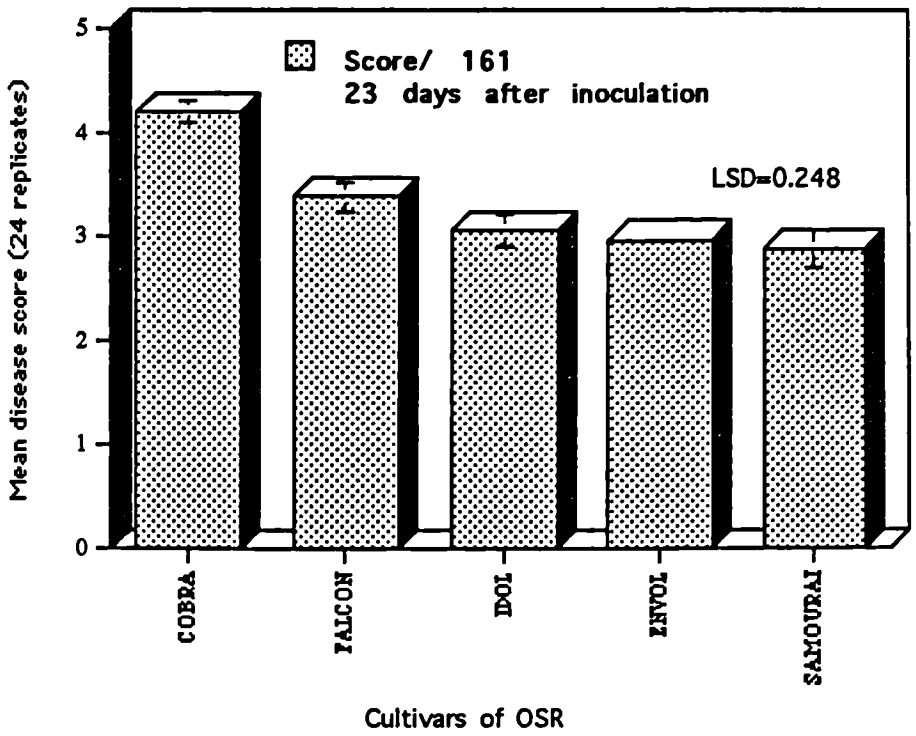


Figure-3.22-Mean disease score of virulent isolate 161 of the pathogenic diploid strain viz.*V.d.longisporum* tested against 5 cvs of OSR 23 days post inoculation. Cobra was the most susceptible cv. of OSR to this isolate with significant differences in susceptibility from all the other cvs at p=0.05. Also Falcon was significantly more virulent than the other 3 cvs (Idol, Envol and Samourai) at p=0.05. There was no significant differences among the susceptibility of the other 3 cvs (Idol, Envol and Samourai) to 161, 23 days post inoculaton (p=0.05, LSD=0.248).

Plate-3.33- Chinese cabbage plants. **A** inoculated with an avirulent haploid isolate 130, (Group A of *V.dahliae*). **B** inoculated with a diploid isolate 161 (Group B of the pathogenic strain viz.*V.d.longisporum*). **C** control plants, one month after inoculation-note stunting of growth and chlorosis of leaves in **B.** (King's College London).

Plate-3.34- Plants of chinese cabbage inoculated with **A**:130, **B**:161, two months after inoculation(see plate-3.33- above for details of isolates). Plant death did not occur even two months after inoculation with the virulent diploid isolate 161 of the pathogenic strain *V.d.longisporum*; however, the extreme reduction of growth and "wilting" of leaves/cotyledons is apparent here (B).

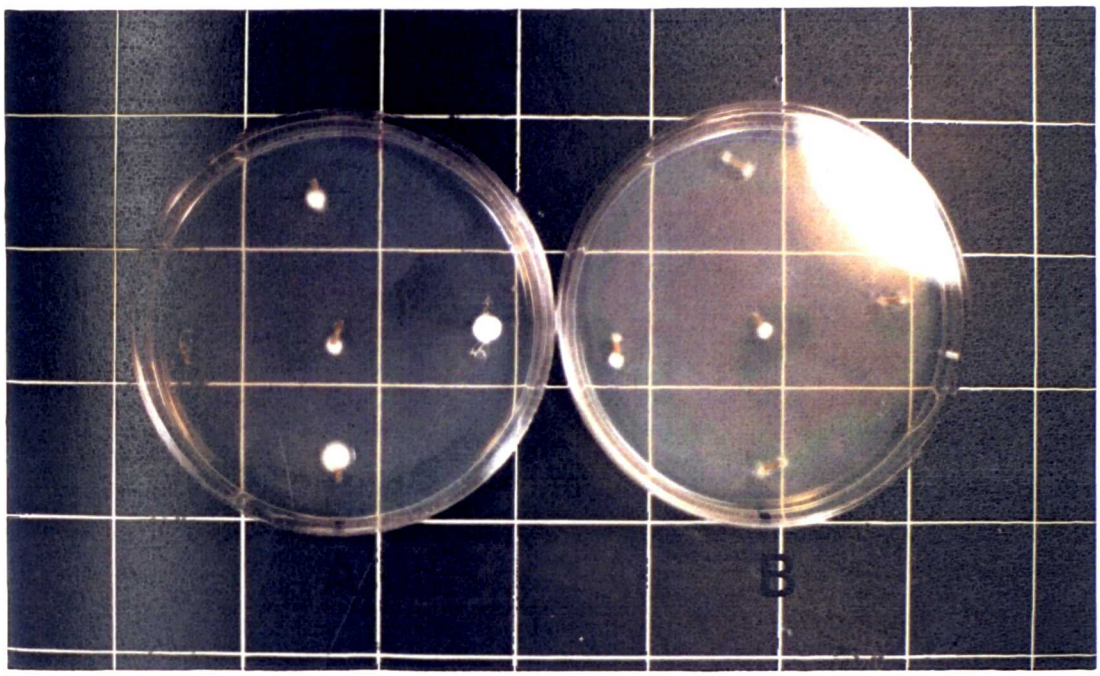


Plate-3.35- Re-isolation of the diploid pathogenic strain viz. *V.d.longisporum* from the stem of two infected OSR seedlings (cv. Cobra) that had been inoculated with two virulent diploid isolates A:161 and B: G10 respectively, 10 days after root-dipping inoculation on re-isolation medium (see section 2.3.5 for details of procedure).

3.1.7.2. Extent of colonization by an avirulent isolate (130) of a non-pathogenic haploid strain (tomato) of *V.dahliae* and a virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum*.

The hypocotyls of seedlings of control OSR plants cv. Cobra and plants inoculated with either a virulent isolate (161) of *V.d.longisporum* (Group B) or an avirulent (tomato) isolate (130) of *V.dahliae* (Group A) were sampled at 6, 9, 13, 15 and 20 days intervals as described in section 2.3.7. Plants were 2 weeks-old at inoculation. The hypocotyl samples were transferred into Petri-dishes containing re-isolation medium in a specific order according to their distance (1, 2, 3, 4 and 5 cm) above the soil surface and were incubated in the dark at 25 ^{0}C for at least 14 days. The recovery of the fungus was calculated for each plant-treatment combination and distance from the soil surface by using the following formula:

(No of plants*/total no of plants) X 100

*No of plants from which the fungus was recovered from the hypocotyls (at the specific distance from the soil surface)

The percentage of hypocotyl colonization for each replicate plant was calculated by using the following formula:

(No of infected segments/5) X 100

At six days post-inoculation, the virulent isolate (161) had reached the lower segments of the hypocotyls, the fungus being recovered from 1, 2 and 3 cm above the soil surface. At 9, 12, 15 and 20 days post-inoculation, the fungus (161) was recovered from all segments (see fig-3.23-) i.e. 100% colonization had occured (see fig-3.24-). The avirulent isolate (130) was recovered from all segments sampled from hypocotyls at only 9 days post-inoculation. After this date, the amount of recoverable fungus (130) in the hypocotyls was reduced to undetectable levels at 20 days post-inoculation (see fig-3.23-). Single factor ANOVA was calculated for % colonization for each day-isolate. There was a highly significant difference in the percentage of hypocotyl colonization between the virulent isolate 161 of the pathogenic diploid strain *V.d.longisporum* and the avirulent isolate (130) of the non-pathogenic (tomato) strain of *V.dahliae* at 9, ($p=0.01$), 13 ($p<0.001$), 15 ($p<0.001$) and 20 days post-inoculation ($p<0.001$). [Data were arcsin transformed before the single factor ANOVA was done].

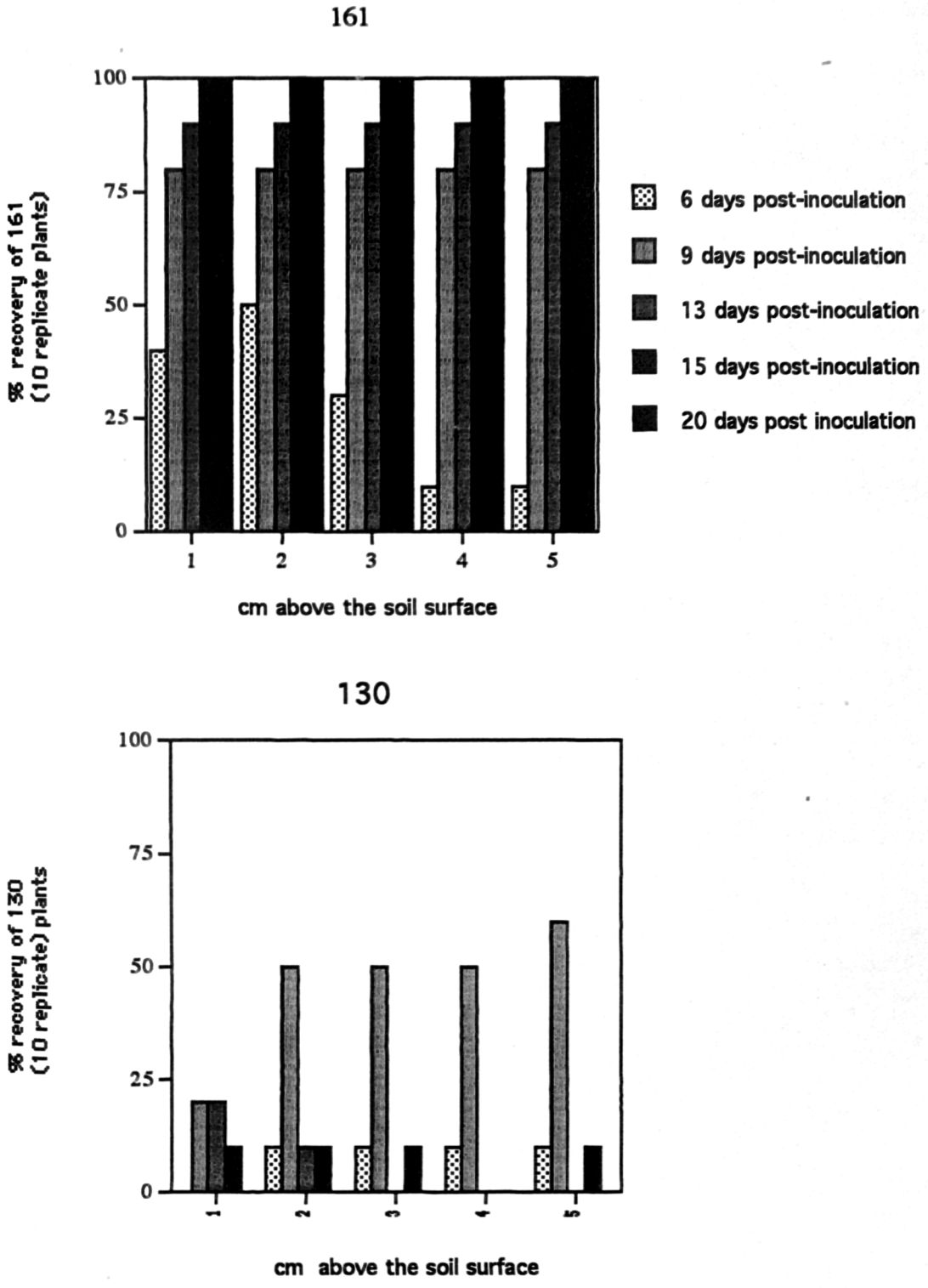


Figure -3.23- Percentage colonization data i.e. % of hypocotyl pieces sampled from which the fungus was recovered (1, 2, 3 ,4, 5 cm hypocotyl segments above the soil surface) at 6, 9, 12, 15 and 20 days post-inoculation. 161 is a virulent isolate of the pathogenic diploid strain viz. *V.d.longisporum* and 130 is an avirulent isolate of a non-pathogenic strain (tomato) of *V.dahliae* .

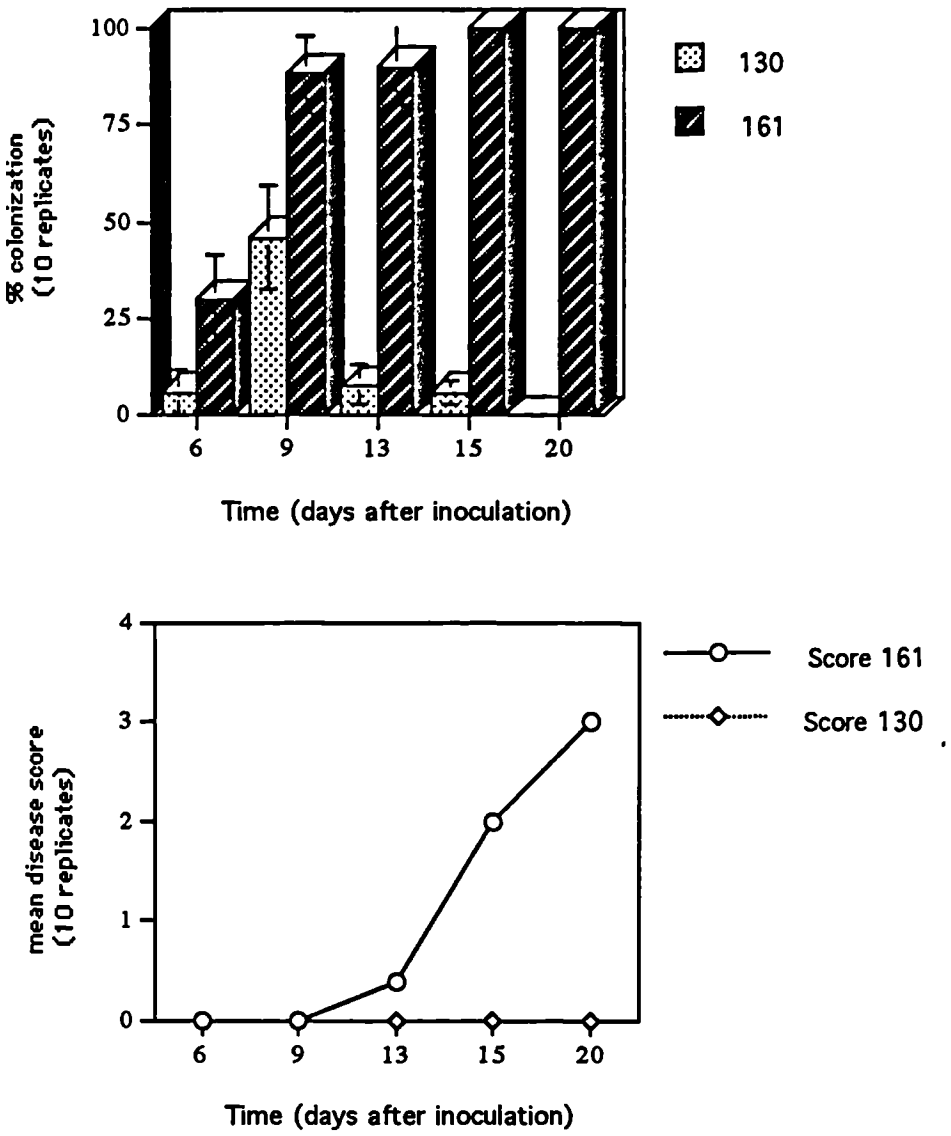


Figure-3.24- Rate of colonization and mean disease scores of OSR cv. Cobra inoculated with either a virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum*, or an avirulent isolate (130) of a non-pathogenic haploid strain (tomato) of *V.dahliae*. Each value is a mean of 10 replicate plants. Each hypocotyl was subdivided into 5 pieces which were transferred into re-isolation medium in a specific order according to the distance above the soil surface (see 2.3.7.). For disease scores assessment see section 2.3.6.

3.1.8. Numerical taxonomy of all isolates tested in this investigation using type of virulence and a combination of morphological, enzymatical and molecular characters.

In previous chapters, a number of different characters (qualitative and quantitative) were used to characterize and classify the different isolates of *V.dahliae*, *V.d.longisporum* and *V.albo-atrum*. Using all these characters, four recombinant strains were detected which showed recombination for the diploidy-associated characters of *V.d.longisporum* (Table -3.30-,-3.31-).

Table-3.30-.*V.dahliae* (n)- and *V.d.longisporum* Stark (2n)-associated characters.

	Virulence to OSR cv. Cobra	Spore length range	Micro-sclerotia	Phialide No	Ploidy Feulgen	Ext. P.p.o	RAPDs-P4a 1.40kb, 1.28kb
HAPLOIDS (8 isolates)	Avirulent	3.5-5.4µm	Compact	4-5	0.7-0.95*	Yes	1.28
DIPLOIDS (27 isolates)	Virulent	7.1-9.1µm	Irregular	3-4	1.2-1.7*	No	1.40

* arbitrary units

Table-3.31-.Four recombinant strains of *V.d.longisporum* detected.

111	SLIGHTLY VIR.	H*	H/D	-	D(2n)	D	D
195	SLIGHTLY VIR.	D#	H	-	D(2n)	D	D/H
G22	AVIR.	H	H	H	H(n)	D	H
G19	SLIGHTLY VIR.	D	H/D	D	H(n)	H	D/H

*H-haploid associated character expressed phenotypically.

#D-diploid associated character expressed phenotypically.

To clearly distinguish the different groups, each character was used as a unit character and the different isolates were used as operational taxonomical units(OTUs). All the data for all unit characters (including the virulence to OSR cv. Cobra) were converted to 1 or 0 for multivariate analysis. Unit characters, which existed in either of two states, e.g. presence or absence of extracellular polyphenol oxidase activity were coded numerically as 1 or 0 corresponding to "+" or "-" respectively. Quantitative characters such as virulence were divided to three multistate characters viz. virulent, slightly virulent and avirulent. Table-3.32- gives the coding for the different characters tested.

Table -3.32-. Coding for different character units tested in the numerical taxonomy exercise for all isolates.

NIT CHARACTERS	PRESENCE/ POSITIVE	ABSENCE/ NEGATIVE
ong spores	1	0
ort spores	1	0
ongate microsclerotia	1	0
herical-Compact microsclerotia	1	0
esting mycelia	1	0
p.p.	1	0
iploidy (shown by Feulgen)	1	0
aploidy (shown by Feulgen)	1	0
irulent to OSR cv. Cobra	1	0
ightly virulent to OSR cv. Cobra	1	0
virulent to OSR cv. Cobra	1	0

Numerical data for all the above characters were combined with the data from RAPDs (presence absence of the 37 bands generated with the 3 primers tested) and were analysed using a UPGMA uster analysis using Jaccard's coefficient, which generated a dendrogram (Figure-3.25-). The data were so analysed by co-ordinate analysis giving a three-dimensional output for the principal clusters. (Figure-26-).

Isolates were clustered into 2 main groups: Cluster of haploid strains of *V.dahliae* and a cluster f the diploid strains viz. *V.d.longisporum*. The two isolates of *V.albo-atrum* (230 from chrysanthemum nd 234 from lucerne) were separated from these two clusters, and from each other. In the first cluster of aploids, the one putative recombinant isolate G22 was somewhat distanced from the other haploid olates in the same cluster. The other 3 putative recombinants (111, 195 and G19) were quite distinct om the other 'normal' diploid isolates in the second main cluster.

Using the co-ordinate analysis isolates, were clearly clustered into 3 main groups. The group A f haploid strains of *V.dahliae*, the group B of the diploid strain of *V.d.longisporum* and group R of the ur recombinants. The other two isolates of V.*albo-atrum* were not in any of these three clusters and gain were distinct from each other.

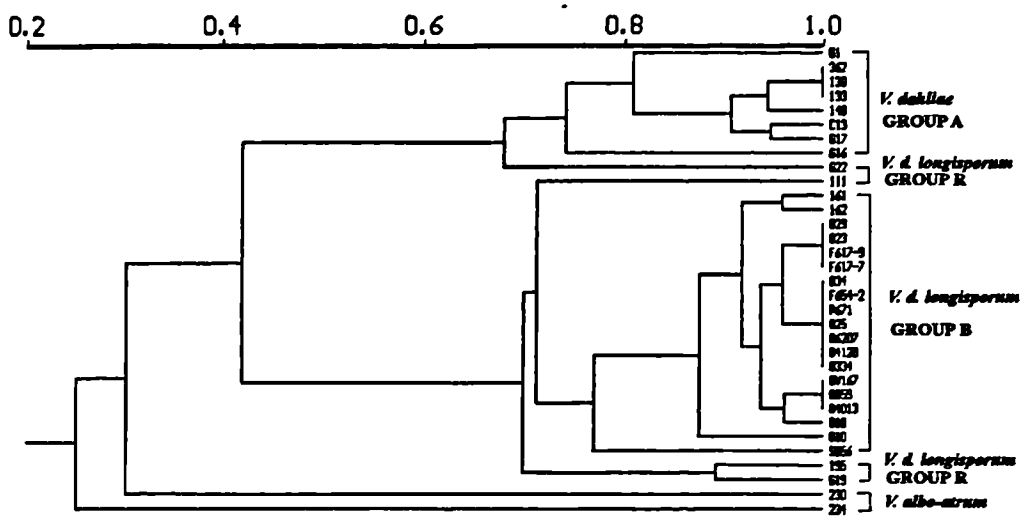


Figure-3.25- Dendrogram derived from UPGMA cluster analysis using Jaccard's coefficient. Dendrogram derived from all characters tested (including RAPDs data) showing relationships among 8 isolates of the haploid strains of *V.dahliae*, 19 isolates of the diploid strain *V.d.longisporum*, 4 putative recombinants of *V.d.longisporum* and two isolates of *V.albo-atrum*. The scale is percentage similarity using Jaccard's similarity coefficient. See table-3.11- for all isolates numbers.

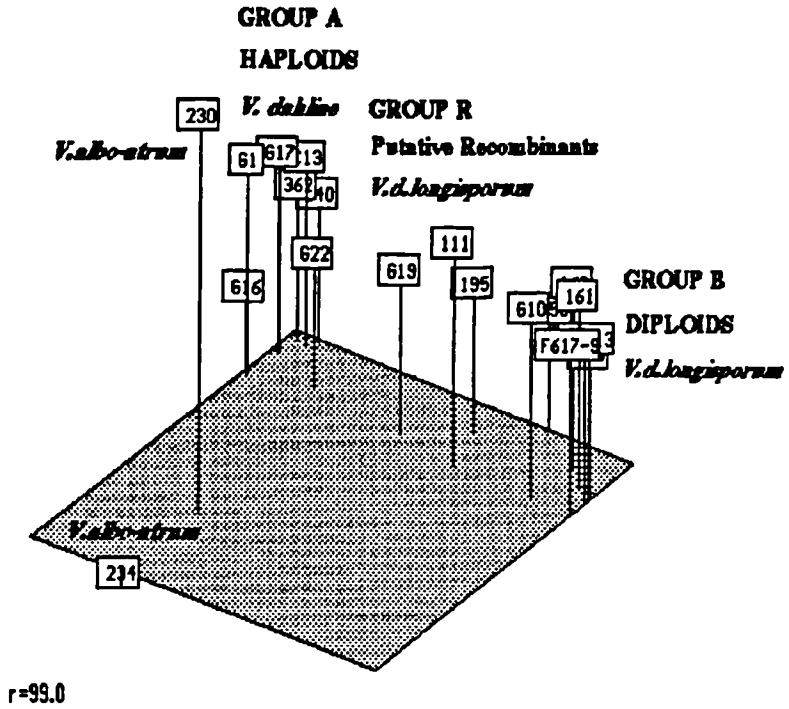


Figure-3.26-.Principle co-ordinate analysis of data for all characters tested, including the RAPDs data, for all isolates in table-3.11-.

3.2. Myrosinase studies

3.2.1. Assays for specific myrosinase activity in extracts of OSR plants (cv.Cobra) inoculated with either a virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum*, or an avirulent isolate (130) of a non-pathogenic haploid strain (tomato) of *V.dahliae* and of control, uninoculated plants (3, 10 and 15 days post-inoculation).

Two week-old OSR seedlings were inoculated using a root-dipping inoculation method as described in 2.4.6. with either the virulent isolate (161) or the avirulent isolate (130). Non-inoculated plants were used as controls.

Tissues (roots, hypocotyls, cotyledons and leaves) of control and inoculated 'pooled' seedlings (20 replicate plants) were extracted in 10mM imidazole-HCL buffer (pH: 6.2) and dialysed against the same buffer as described in section 2.5.1.1. The amount of protein in the desalted extract was determined from a plotted standard curve: OD_{595} versus standard concentration of BSA in imidazole-HCL buffer pH:6.2 .A standard curve was prepared from standard concentrations of BSA (1-20µg/ml) each time the assays were performed. Figure -3.27- gives a typical standard curve for the microassay procedure.

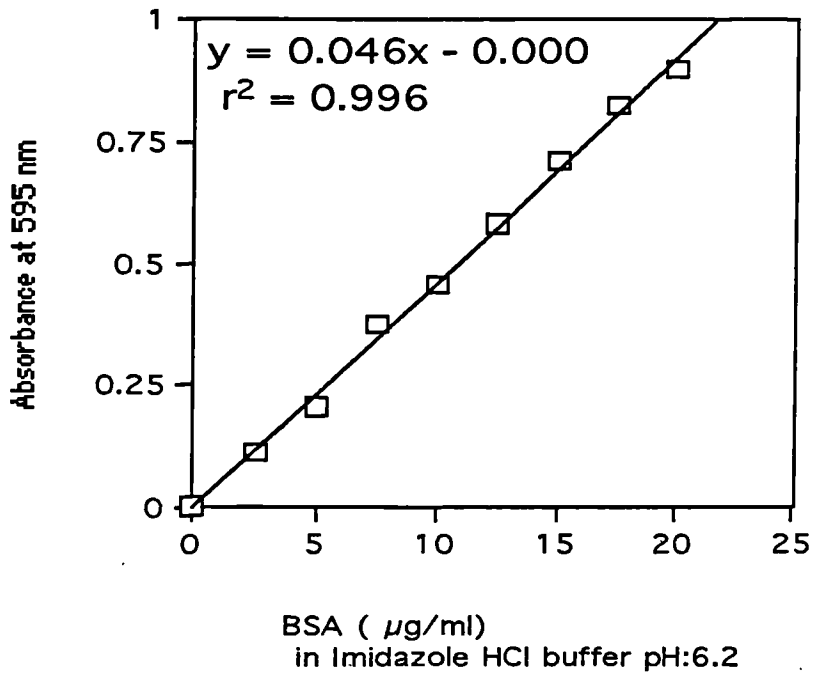


Figure-3.27- Protein determination: Typical standard curve for the Biorad-Protein microassay of 1-20µg/ml Bovine serum albumin (BSA) in imidazole-HCL buffer pH:6.2. Each value is the mean of three different determinations at 595nm.

The myrosinase assays were conducted in the presence of ascorbic acid (0.3mM) in Na acetate (0.1M) buffer at pH 5.5 (James & Rossiter,1991).[Ascorbic acid exerts an activating effect on plant myrosinases (Ohtsuru & Hata,1979)] .

The specific activity of the desalted enzyme (units[1] /mg desalted protein) was determined by a glucose release assay using a Sigma glucose kit based on the hexokinase reaction as described in section 2.5.1.5. Initially, the GOD-Perid reagent (Boehringer) was used for the determination of glucose, but ascorbic acid was found to interfere with the ABTS[2] present in the reagent which was responsible for the colour complex detected by spectrophotometer,thus giving inconsistent spectrophotometric values.

In preliminary experiments, myrosinase was extracted from roots excised from uninoculated and inoculated plants(avirulent isolate 130 and virulent isolate 161) at 1, 3 and 4 days post-inoculation. The values obtained for the specific activity of myrosinase in extracts from inoculated tissue were found to be higher than those of control extracts only at 3 days post-inoculation (see figure-3.28-).

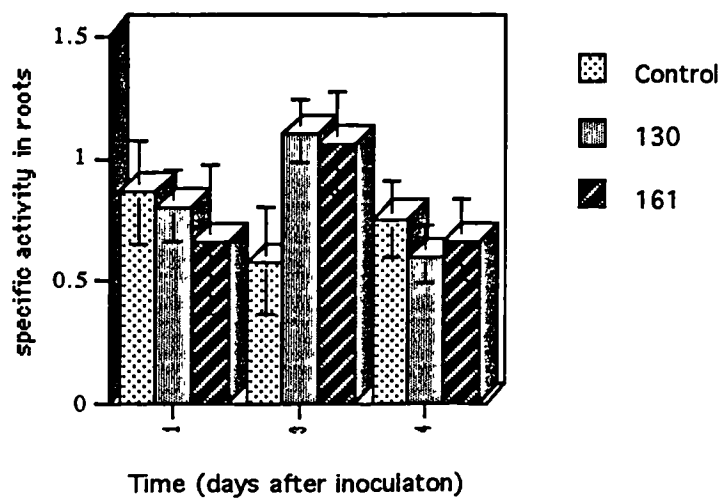


Figure-3.28- Specific myrosinase activity (µmole glucose released /min/mg protein) in extracts from roots of OSR cv. Cobra from control uninoculated plants, plants inoculated* with an avirulent isolate (130) of a non-pathogenic strain (tomato) of *V.dahliae* or a virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum* at 1, 3 and 4 days post-inoculation. Glucose release assays were performed in the presence of ascorbic acid (0.3mM). Each value is a mean of 3 extractions of 20 'pooled' roots each. Bars represent standard errors of the means.

* plants inoculated by root-dipping at 2 weeks old.

[1] The amount of enzyme that catalyses the formation of 1µmoles glucose /min under the defined conditions.
[2] di-ammonium 2,2'-azino-bis

To confirm this result, extractions were performed from 'pooled' twenty plants for each treatment at 3 days post-inoculation (5 replicate extractions of 20 plants) and again the values for the specific activity of myrosinase were higher from roots inoculated with either isolate 130 or 161, as compared with the specific activity of the control, uninoculated plants (see fig-3.29-). There was a highly significant increase in specific myrosinase activity in root tissue extracts for both 130- and 161-inoculated plants as compared with control plants ($p<0.05$).

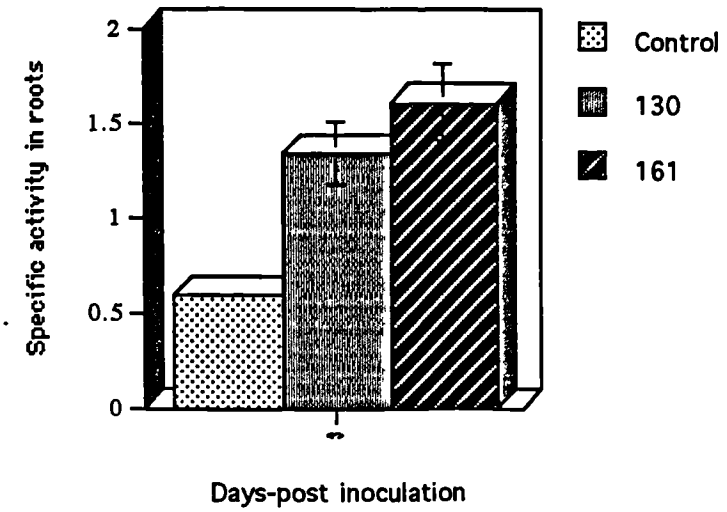


Figure-3.29- Specific myrosinase activity (µmole glucose released /min/mg protein) in roots of OSR cv. Cobra from control uninoculated plants, plants inoculated* with an avirulent isolate (130) of a non-pathogenic strain (tomato) of *V.dahliae* or a virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum* at 3 days post-inoculation. Glucose release assays were performed in the presence of ascorbic acid (0.3mM). Each value is a mean of 5 replicate extractions of 20 pooled roots each. Bars represent standard errors of the means.
* plants inoculated at 2 weeks-old by root-dipping.

To further investigate the activation of the enzyme myrosinase as a response to inoculation, a time course study was performed at 3, 10 and 15 days post inoculation. Two week-old seedlings were inoculated by root-dipping inoculation as described in 2.4.6. with either a virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum* or an avirulent isolate (130) of a non-pathogenic strain (tomato) of *V.dahliae* . Non-inoculated plants were used as controls. Twenty individual plants were used and pooled for extractions for each treatment and the experiment was repeated twice. Myrosinase from roots, hypocotyls, cotyledons and leaves were assessed separately for each tissue and for each treatment. The extractions were performed as described in section 2.5.1.1. The specific activity of the desalted myrosinase was determined in sodium acetate buffer (pH=5.5) in the presence of ascorbic acid (0.3 mM) by a glucose release assay as described in section 2.5.1.5.

3.2.1.1. Myrosinase activity in roots of uninoculated and inoculated OSR plants (cv. Cobra)

The specific activity in roots of plants inoculated at 2 weeks-old with either the virulent isolate (161) or the avirulent isolate (130) was markedly higher as compared with the control, uninoculated plants at 3 days post-inoculation, but by 10 and 15 days post-inoculation it had decreased and increased respectively to control levels (see figure-3.30-). So there was a large initial increase by 3 days post-inoculation for both 161 and 130 inoculated plants confirming the earlier results.

The specific myrosinase activity in roots of control, healthy (uninoculated) plants of OSR was found to increase during growth, reaching the max. value at 15 days post-inoculation.(see figure-3.30-). On the contrary its activity in roots inoculated with either a virulent isolate (161) or an avirulent isolate (130) had decreased to control levels by 10 days post-inoculation and then increased by 15 days post-inoculation for both inoculated treatments again reaching similar levels to the controls.

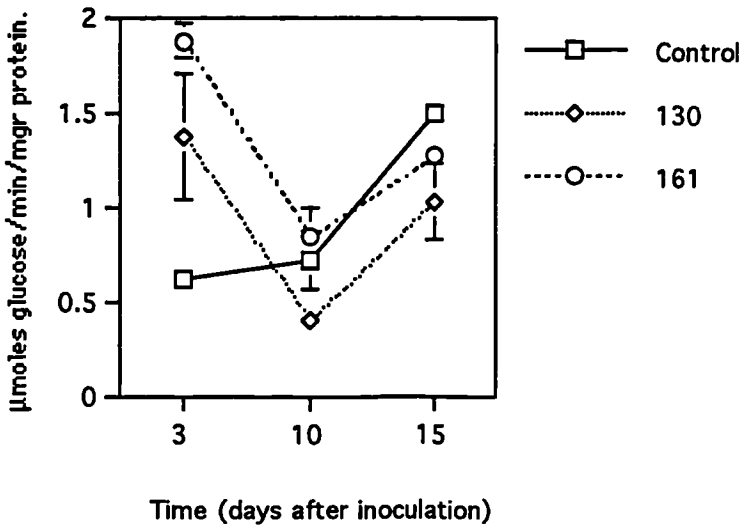


Figure-3.30-Specific myrosinase activity (µmole glucose /min/mg protein) in roots of control uninoculated plants and plants inoculated* with either an avirulent isolate (130) of a non-pathogenic strain (tomato) of *V.dahliae*, or a virulent isolate (161) of the pathogenic strain viz. *V.d.longisporum*. Each value is the mean of three different extractions of 20 'pooled' roots each and bars represent S.E..

*plants inoculated at 2 weeks by root dipping.

3.2.1.2. Myrosinase activity in hypocotyls of uninoculated and inoculated OSR plants (cv. Cobra)

The specific myrosinase activity in hypocotyls in control, uninoculated plants slightly decreased during growth, whereas in plants inoculated with the virulent isolate (161), it decreased at 10 days post-inoculation, finally increasing again to control levels by 15 days-post inoculation. On the contrary, the specific myrosinase activity in hypocotyls of the plants inoculated with the avirulent isolate (130) increased

markedly by 10 days post-inoculation as compared with the activity in hypocotyls in control plants, and slightly decreased by 15 days post-inoculation (see figure-3.31-).

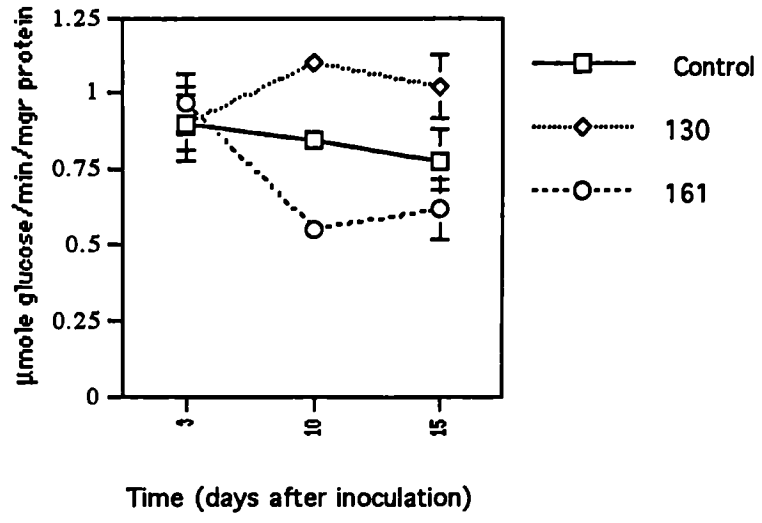


Figure-3.31-Specific myrosinase activity (µmole glucose /min/mg protein) in hypocotyls of control, uninoculated plants, and plants inoculated* with either an avirulent isolate (130) of a non-pathogenic strain (tomato) of *V.dahliae* or a virulent isolate (161) of the pathogenic strain viz. *V.d.longisporum*. Each value is the mean of three different extractions of 20 'pooled' hypocotyls each and bars represent S.E. *Plants were 2 weeks-old at inoculation.

3.2.1.3. Myrosinase activity in cotyledons of uninoculated and inoculated OSR plants (cv. Cobra).

The specific myrosinase activity in cotyledons for all treatments was only detectable at 3 and 10 days post inoculation, and it decreased almost 10 times by 10 days post-inoculation for all treatments. The specific activity in cotyledons of plants inoculated with the virulent isolate (161) was higher at 3 days post-inoculation as compared with that of "130-inoculated" plants (see figure-3.32-).

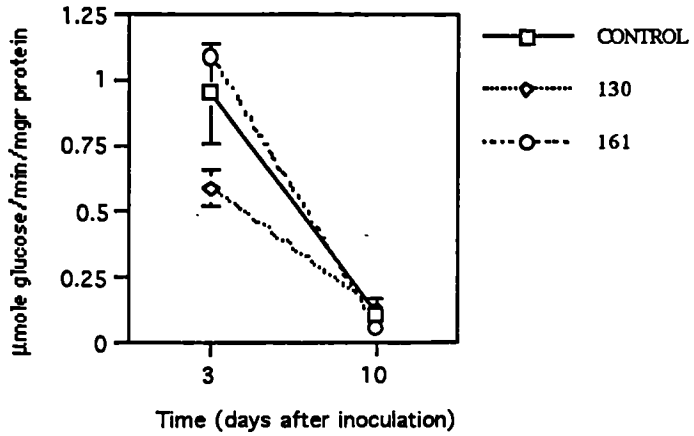


Figure-3.32-Specific myrosinase activity (µmole glucose /min/mg protein) in cotyledons of control, uninoculated plants, and plants inoculated* with either an avirulent isolate (130) of a non-pathogenic strain (tomato)of *V.dahliae* or a virulent isolate (161) of the pathogenic strain viz.*V.d.longisporum*. Each value is the mean of three different extractions of 'pooled' cotyledons of 20 plants each and bars represent S.E.*Plants were 2 weeks old at inoculation by root-dipping.

3.2.1.4. Myrosinase activity in leaves of uninoculated and inoculated OSR plants (cv. Cobra)

Myrosinase activity in leaves of control and inoculated plants (130,161) was only detected 3 and 10 days post-inoculation. The specific activity decreased almost 10 times during growth for the leaves of control plants (see figure-3.33-). The specific activity in the leaves of plants inoculated with the virulent isolate (161) was lower as compared with the specific activity in the leaves of plants inoculated with the avirulent isolate (130) or control uninoculated plants (see figure-3.33-)

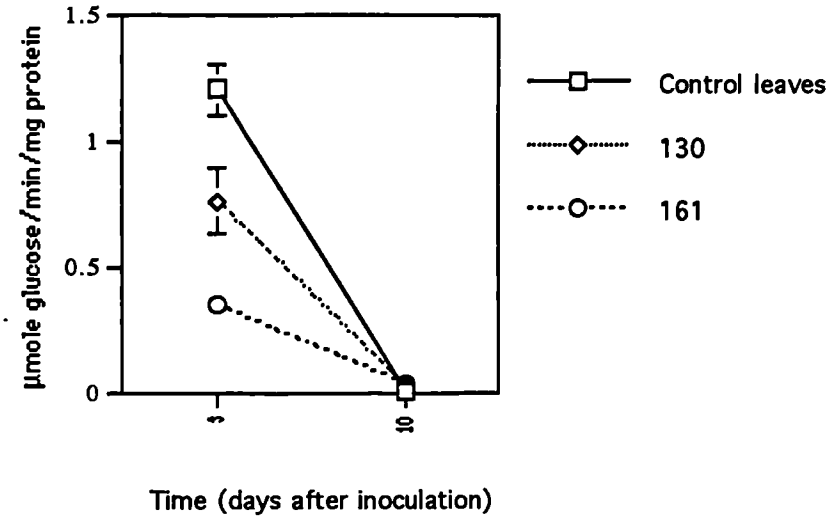


Figure-3.33-Specific myrosinase activity (µmole glucose /min/mg protein) in leaves of control, uninoculated plants, and plants inoculated with either an avirulent isolate (130) of a non-pathogenic strain (tomato) of *V.dahliae*, or a virulent isolate (161) of the pathogenic strain viz.*V.d.longisporum*. Each value is the mean of three different extractions of 'pooled' leaves of 20 plants and bars represent S.E. Plants were 2 weeks-old when inoculated by root-dipping.

3.2.1.5. Myrosinase activity in petioles of leaves of control uninoculated plants and inoculated OSR plants (cv. Cobra).

Because myrosinase activity in whole leaf extracts was almost undetectable even at 10 days after inoculation, myrosinase was extracted from petioles of control plants and from petioles of plants that had been inoculated with either virulent isolate 161 or the avirulent isolate 130 at 20 days post-inoculation. The specific activity in the petioles of the infected leaves of plants that had been inoculated with the virulent isolate 161 was markedly higher than the specific activity for healthy controls or "130-inoculated" plants (see figure-3.34-).

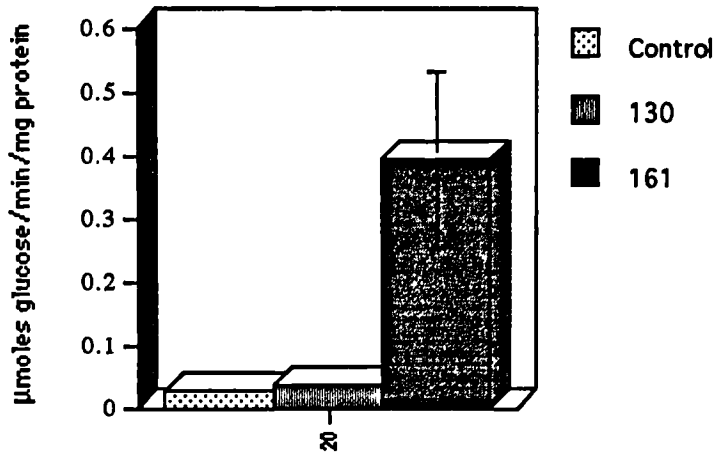


Figure-3.34- Specific myrosinase activity (glucose /min/mg protein) at 20 days after inoculation in petioles of leaves of control uninoculated plants and plants inoculated with either an avirulent isolate (130) of a non-pathogenic strain of *V.dahliae* (tomato) or a virulent isolate (161) of the pathogenic strain viz.*V.d.longisporum*. Each value is the mean of three different extractions of 20 pooled individual petioles for each treatment and bars represent S.E. of the means. Plants were 2 weeks-old when inoculated by root-dipping.

3.2.2 Localization of myrosinase

3.2.2.1. Histochemical localization of myrosinase in hand-cut sections of radicles

Initially, myrosin cells were localized in hand-cut sections of radicles of OSR cv. Cobra and *Raphanus sativus* by the presence of black, barium sulphate deposits at the site of myrosinase hydrolysis of the substrate sinigrin in the presence of barium chloride. Myrosin cells were detected as being densely blue after staining with lactophenol cotton blue, as compared with the surrounding cells; when sinigrin was included in the reaction, black deposits (being the product of the enzymatic breakdown of sinigrin) appeared in the same myrosin cells (see Plate-3.37-). In the control sections with no sinigrin in the reaction, there were no black deposits. Myrosin cells were detected 24, 48 and 72h post-imbibition. After 72h, the staining was less dense and it was very difficult to obtain hand-cut sections. Myrosin cells were detected in the outer-cortex of the embryonic axis of both OSR (see Plate-3.36-) and *Raphanus sativus* radicles.

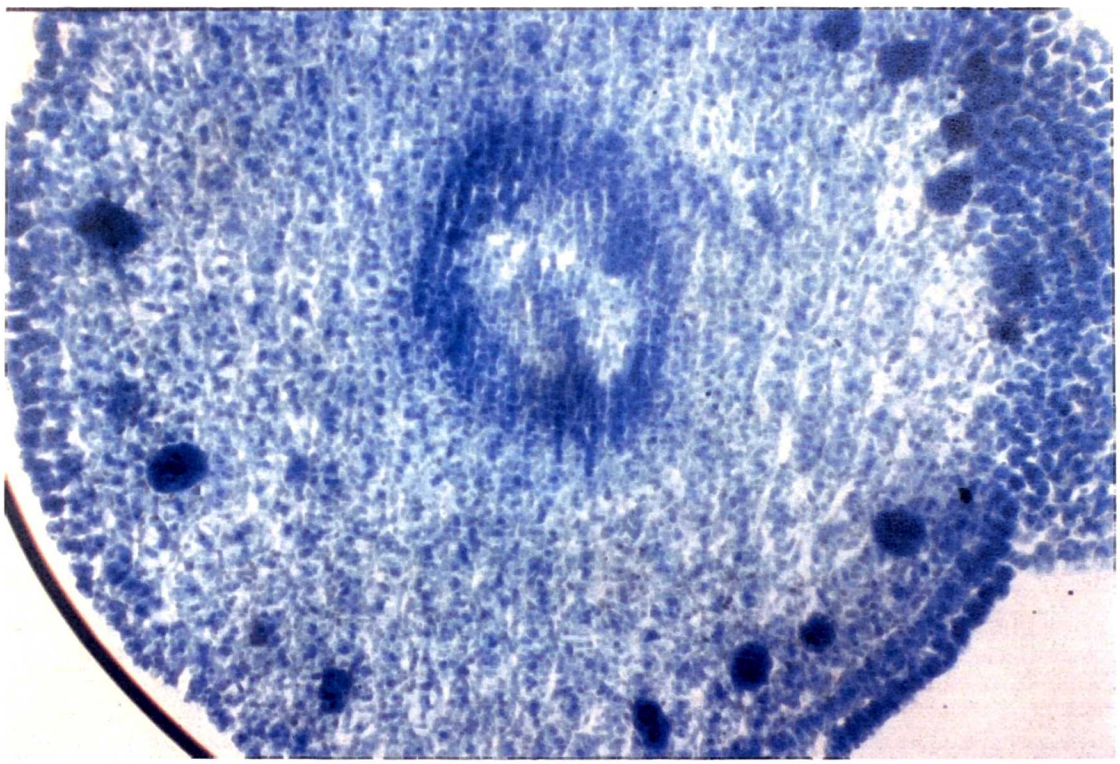


Plate-3.36- Myrosin cells in the outer cortex of the embryonic axis of OSR cv. Cobra 24 h after imbibition. Hand-cut sections were incubated in the presence of sinigrin, barium chloride and ascorbic acid, stained with lactophenol cotton blue followed by clearing in lactophenol and viewed under the light microscope(X125).

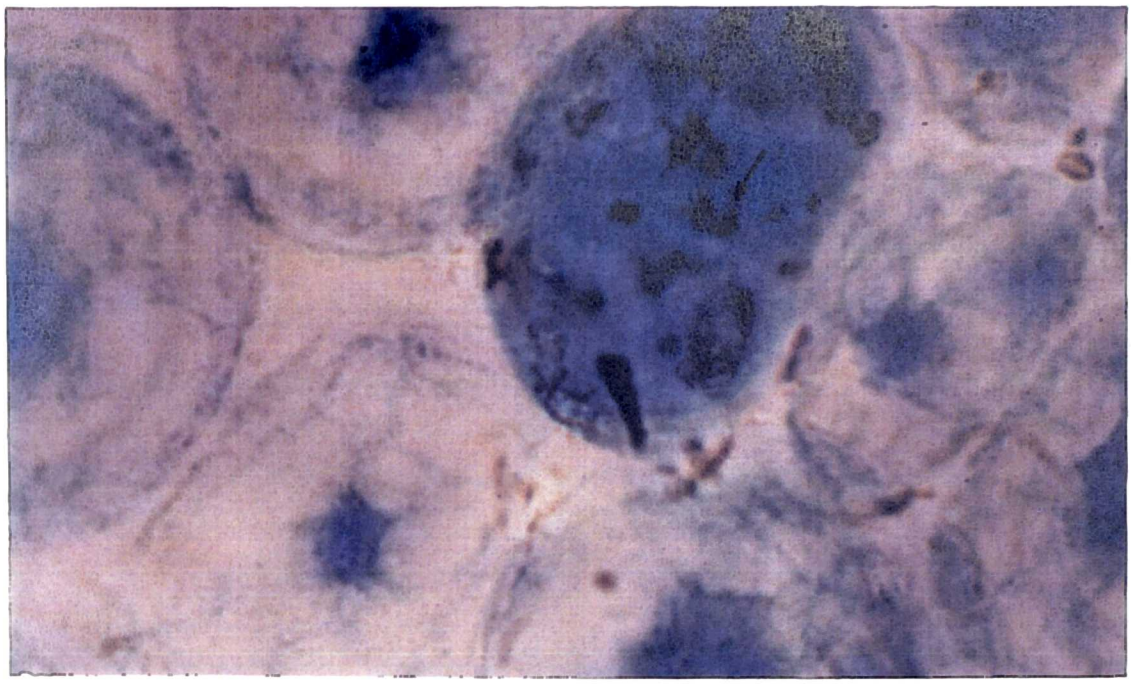


Plate-3.37- Myrosin cell of the radicle of OSR, 24h after imbibition. Black deposits (arrow) of barium sulphate, product of sinigrin breakdown hydrolysis by myrosinase, can be detected. Hand-cut sections were incubated in the presence of sinigrin, barium chloride and ascorbic acid, stained with lactophenol cotton blue, followed by clearing in lactophenol and viewed under the light microscope (X1250).

3.2.2.2. Immunogold localization of myrosinase, using two different polyclonal antibodies (M1, KO89) raised against myrosinase, in ultra-thin sections of chemically-fixed radicles and cotyledons.

3.2.2.2.1. In cotyledons of OSR cv. Cobra

Cotyledons of seedlings of OSR (cv. Cobra) that had been germinated in water for 72h were prepared for electron microscopy (both conventional and that involving immunogold labelling as described in section 2.5.2.2.2. (fixation a, and immunogold labelling procedure 1, page 108). The primary antibody M1 was used in a 1/1000 dilution. Sections were stained with anti-myrosinase and 100nm colloidal gold.

For light microscopy studies the resin blocks containing the tissue were thick sectioned (1-2μm sections) and stained with 1% toluidine in 0.5% Borax. The tissue was examined for myrosin cells, which stained more intensively blue when compared with the other cotyledon cells. Myrosin cells were scattered in the mesophyll tissue of the cotyledons. No other cells were stained as intensively blue as myrosin cells. This stain is not a specific one, but it allowed the identification and exact location of myrosin cells, and so the area of interest was trimmed down for the cutting of ultra-thin sections for electron microscopy studies (100nm sections).

Ultra structural EM studies showed that myrosinase was localized in specific cells (myrosin cells) in myrosin grains (mg). These cells were of different shape, ie. elongate (Plate-3.38-) or spherical (Plate-3.39-), and were scattered through the other mesophyll cells. The other mesophyll cells were dominated by a very large vacuole (Plate-3.42). No labelling was found in any organelle or membrane of these other cells (Plate-3.40-,-3.43-). Within myrosin cells no labelling was found in cytoplasm, oilbodies, plasma membrane, cell walls, nucleus, and mitochondria.Thus colloidal gold label was restricted to myrosin grains in myrosin cells (Plates -3.40, -3.41-, -3.44-, -3.45, -3.46-).

Myrosin cells were occupied by one-three, large, myrosin grains (Plate-3.44-) or/and a number of smaller ones (Plate-3.41-). In myrosin grains, gold particles were always associated with the electron-opaque area of the grain (Plate-3.45-). Electron translucent areas inside the grain demonstrated lack of the enzyme (Plate-3.46-).

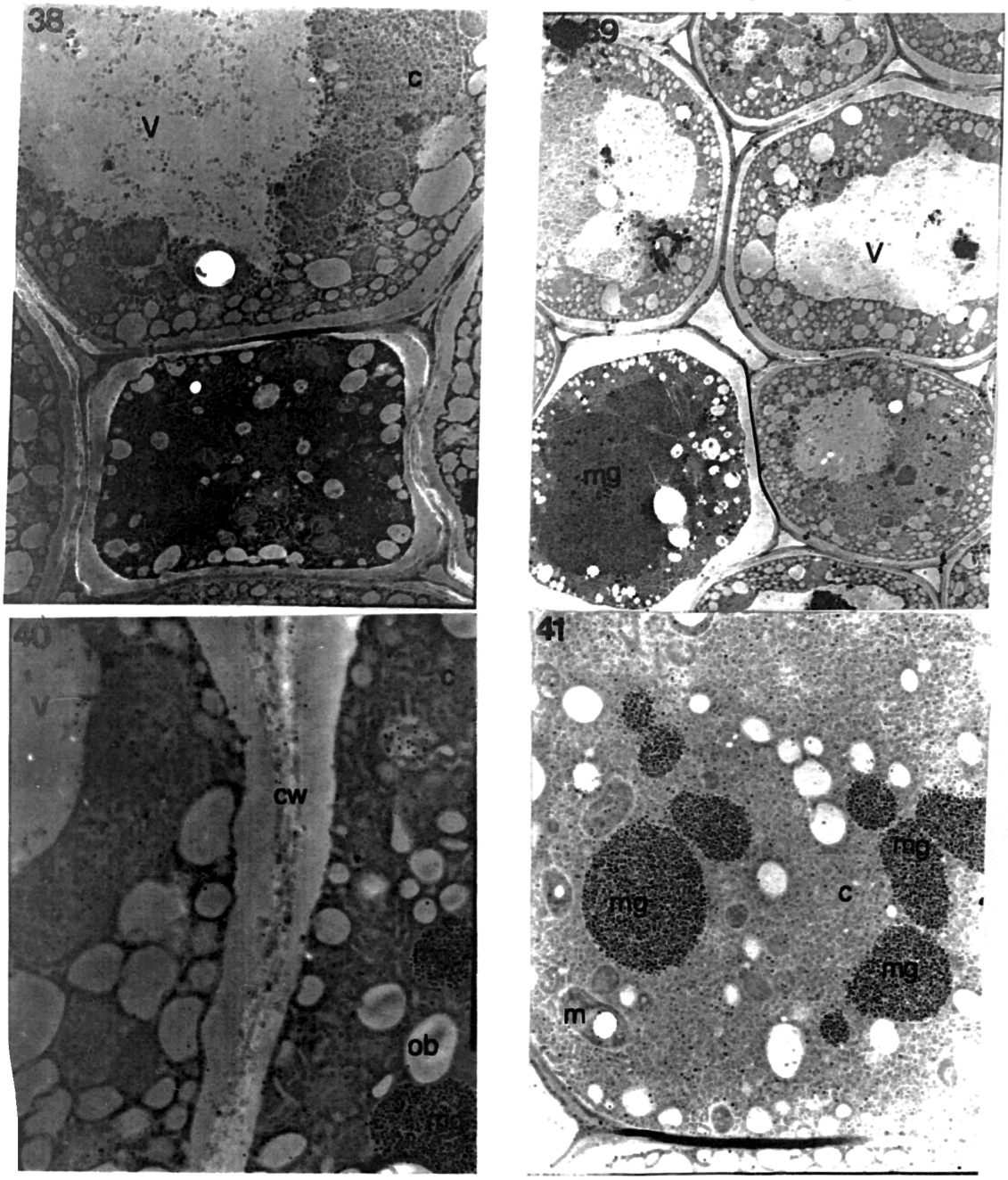
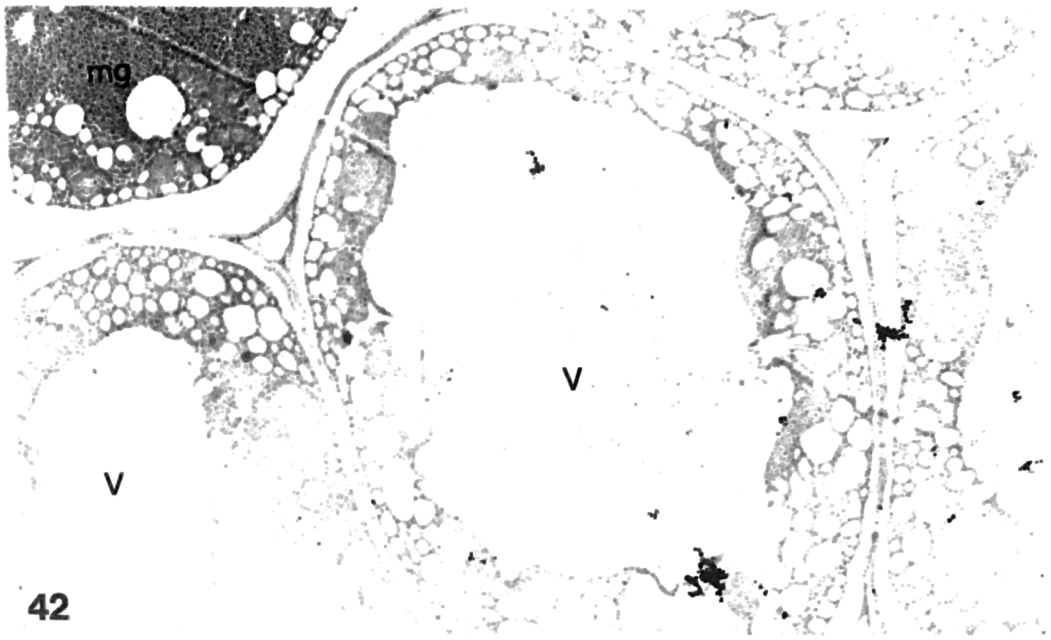


Plate -3.38- Immunogold labelling of cotyledon mesophyll cells of oilseed rape cv. Cobra. One irregular myrosin cell can be seen with a number of myrosin grains (mg). The other mesophyll cell is occupied by a very large vacuole (V) which showed no positive reaction to the antibody. The section was treated with anti-myrosinase and 100nm colloidal gold. (Magnification X 5K).

Plate-3.39- One spherical myrosin cell in mesophyll tissue of the cotyledon of oilseed rape cv. Cobra. Myrosinase is restricted to the myrosin grains (mg) of the myrosin cell. V: vacuole. (Magnification X2K).

Plate-3.40- Myrosin cell adjacent to a mesophyll cotyledon cell. The label can be seen only in myrosin grains (mg) in the myrosin cell. Oil bodies (ob), cell walls (cw) and vacuole (V) lack the enzyme. (Magnification X12K).

Plate-3.41- A number of small myrosin grains (mg) can be seen in a myrosin cell in a cotyledon of oilseed rape cv. Cobra. Cytoplasm (c) and mitochondria (m) show a negative reaction. (Magnification X 8K).



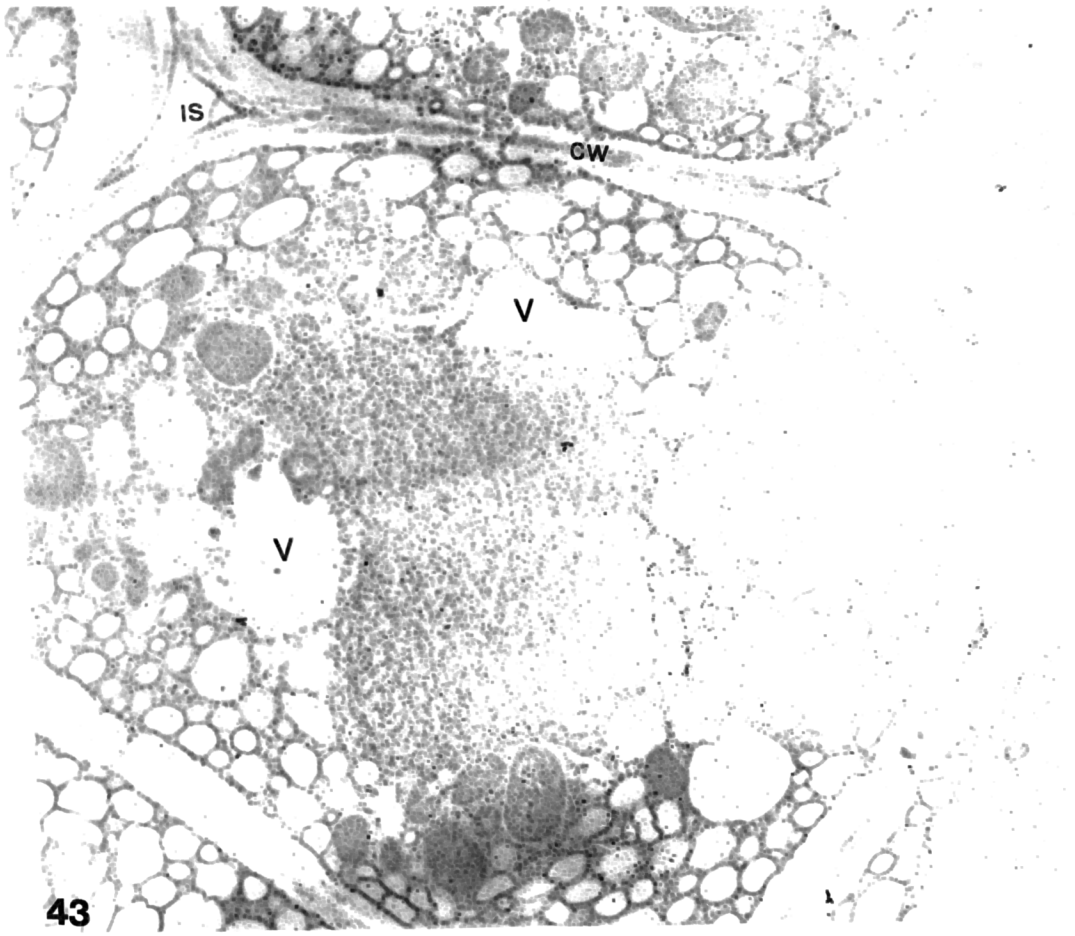
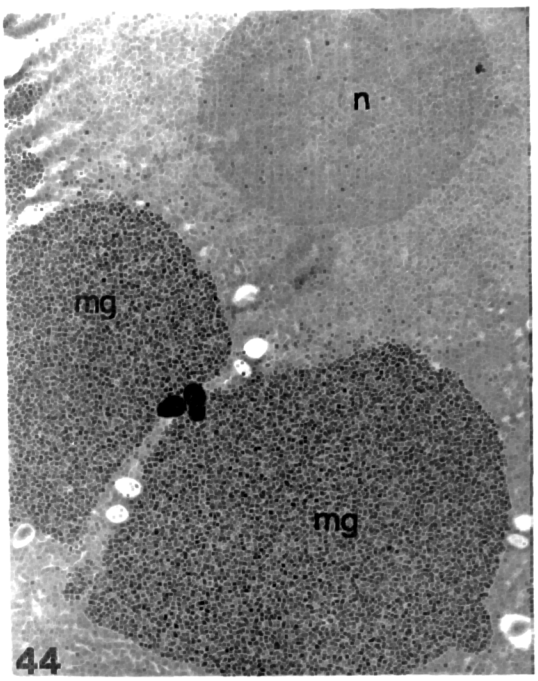
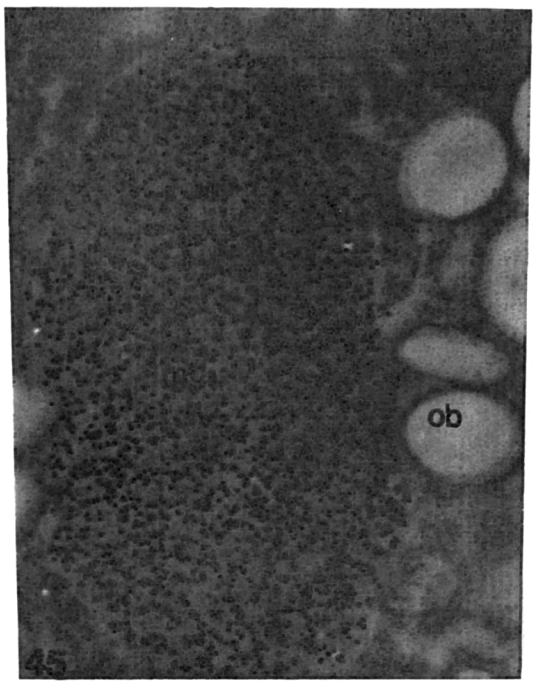


Plate-3.42- Mesophyll cotyledon cells with empty vacuoles (V). Myrosinase is localized only in myrosin grains (mg) in myrosin cells. (Magnification X5K)

Plate-3.43- No label can be seen in any organelle or membrane of these other mesophyll cells. Cell wall (c), Intercellular space (is), vacuole (V).(Magnification X10K).





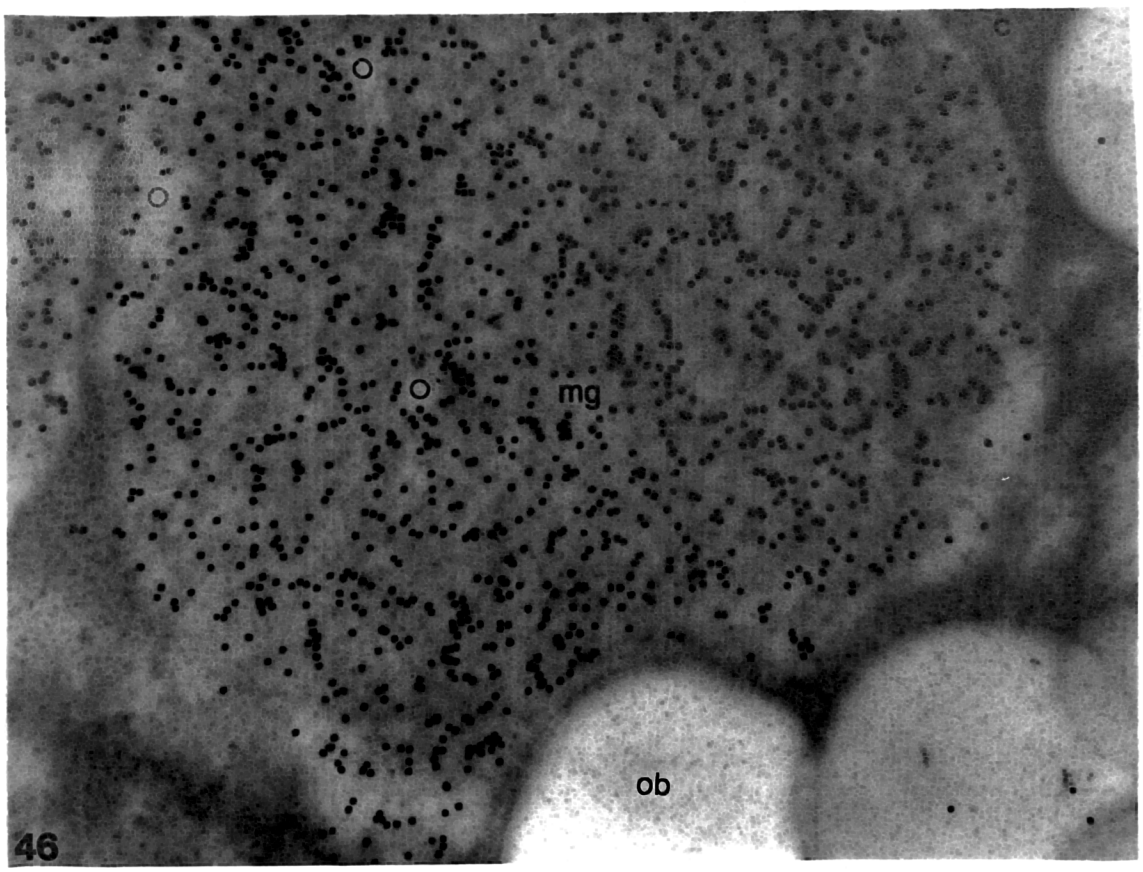


Plate -3.44- One myrosin cell with two large myrosin grains. The myrosinase is restricted to the grains. No label can be seen in the nucleus (n). (Magnification X8K).

Plate-3.45- One myrosin grain with positive labelling and oilbodies with no labelling. (Magnification X40K).

Plate-3.46- Detail of a myrosin grain (mg). Myrosinase is associated with the electron-opaque areas of the myrosin grain. Electron translucent areas inside the grain demonstrate lack of label (circle). No label can be seen in cytoplasm (c) or oil bodies (ob). Magnification (X80K).

3.2.2.2.2. In radicles of OSR cv. Cobra.

Radicles of OSR (cv. Cobra) that had been germinated in water for 48h were inoculated with a drop (20µl) of spore suspension ($1X10^6$ spores/ml) of either the virulent isolate 161 of the pathogenic diploid strain viz. *V.d.longisporum* or the avirulent isolate 130 of a non-pathogenic haploid strain (tomato) of *V.dahliae* . Control radicles were inoculated with a 20µl of sterile distilled water. After 24 h, samples were prepared for electron microscopy (both conventional and that involving immunogold labelling) as described in section 2.5.2.2.2 (fixation b, and immunogold labelling procedure 2, page 109). The primary antibody KO89 was used in a 1/15.000 dilution.

For light microscopy studies, the resin blocks containing the tissue were thick sectioned (1-2µm thick) and stained with 1% toluidine blue in 0.5% Borax. The tissue was examined for myrosin cells, which stained more intensively blue when compared with the other radicle cells. These myrosin cells were scattered in the outer cortical cells of the radicle and contained myrosin grains that occupied almost the entire cell (1 or 2 or 3 grains /cell). No other cells were stained as intensively blue as myrosin cells. This stain is not a specific one, but it allowed the identification and exact location of myrosin cells; thus, the area of interest was trimmed down for the cutting of ultrathin sections for electron microscopy studies (100nm sections). No hyphae of either the virulent isolate 161, or the avirulent isolate 130, were observed in the inoculated radicles, but areas of tissue with myrosin cells were trimmed and prepared for EM studies for both inoculated-treatments.

Immunogold labelling of sections showed a specific positive localization of myrosinase, mainly in the interior of myrosin grains in the myrosin cells (Plate-3.47-, -3.51-). No such labelling was found in the cell walls(cw), plasma membrane (pm), oil bodies (ob), dilated cisternae of the endoplastic reticulum or the cytoplasm of myrosin cells (Plate-3.52-). One-three, very large, myrosin grains were found in each myrosin cell, but also a number of small myrosin grains were distributed inside the cytoplasm of the myrosin cell (Plate-3.48-). Inside the myrosin grains, the gold particles were distributed uniformly (Plate-3.48-). No degradation of myrosin cells was observed at this stage of development(72h germinated radicle). On the contrary, aleurone grains had been degraded at this developmental stage, and the cells were occupied by empty vacuoles, or vacuoles with electron opaque deposits at the periphery or inside, the vacuole (Plate-3.53-,-3.56-). These electron opaque deposits also showed a positive reaction to the antibody, but to a lesser extent (Plate-3.54-). There was no labelling in the cytoplasm of either myrosin or aleurone cells (Plate-3.50-).

Radicles inoculated with either the virulent isolate 161 or the avirulent isolate 130 showed the same reaction as the controls.(i.e. specific labelling in myrosin grains, within myrosin cells). No hyphae were detected in association with myrosin cells or aleurone cells. However, some myrosin cells in radicles that had been inoculated with the virulent isolate 161 showed a less dense matrix in the myrosin grains (Figure-3.49-).

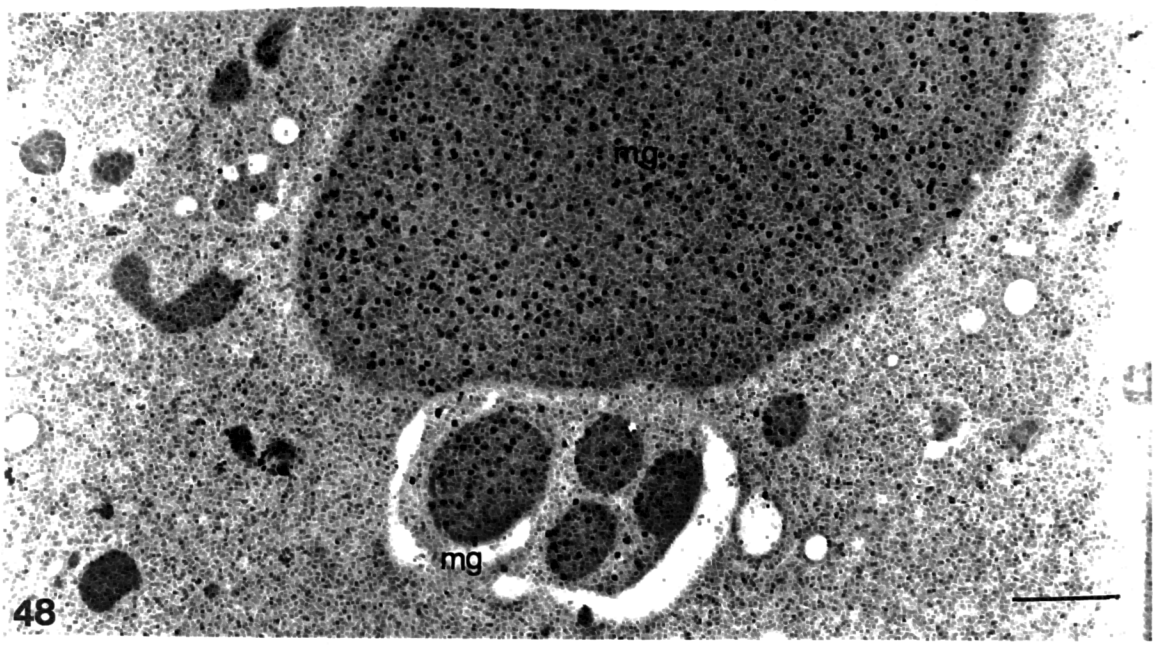
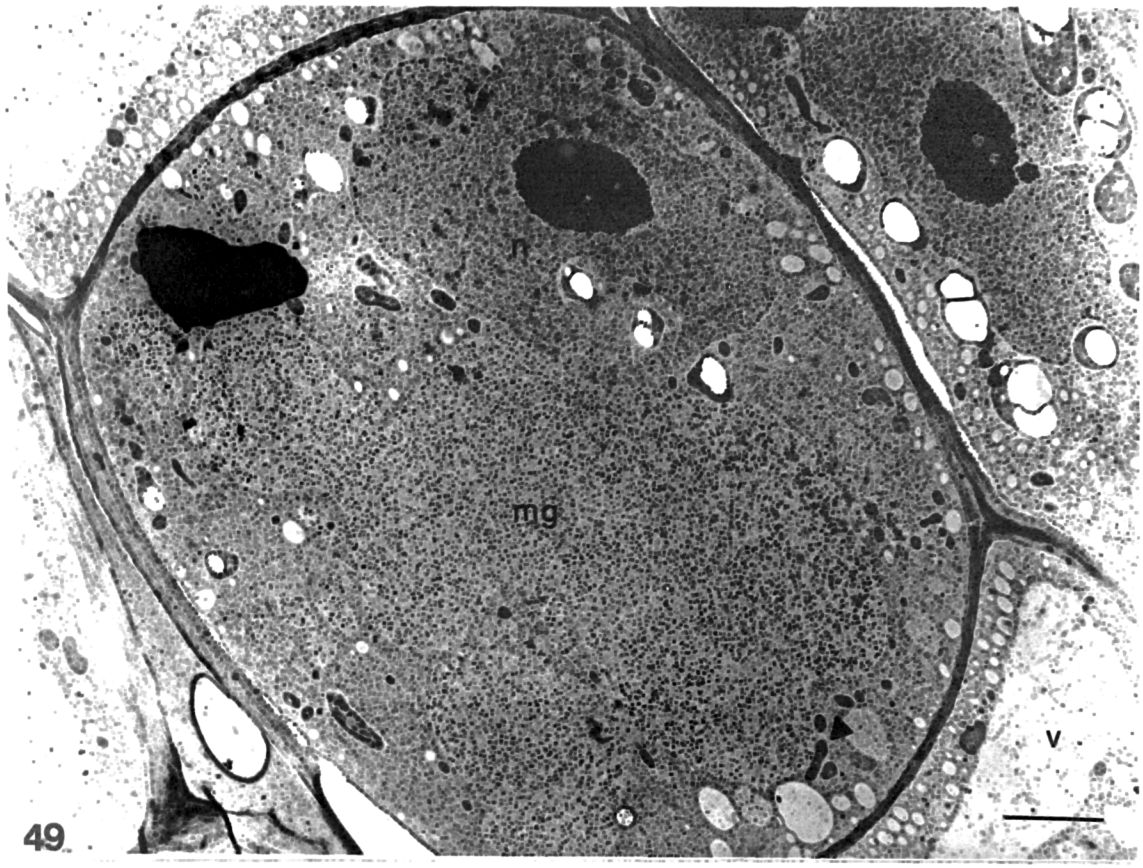


Plate-3.47- Immunogold localization of myrosinase in a *Brassica napus* radicle (control). Myrosinase was localized inside the myrosin grains (mg) in myrosin cells. One very large grain can be seen and a number of smaller ones. No such labelling can been seen on the plasma membrane (pm), cell walls (cw), cytoplasm (c) or in other cytoplasmic organelles i.e. mitochondria (m). The section was labelled with anti-myrosinase antibody (K089) and 30nm colloidal gold, then post-stained with 1% osmium tetroxide. The section was examined with a Jeol 1200 EX (Japan) electron microscope at 60 V. Bar: 2 µm.

Plate-3.48- Detail of one myrosin grain, revealing the uniform internal distribution of the immunogold-labelled myrosinase. Bar: 1µm



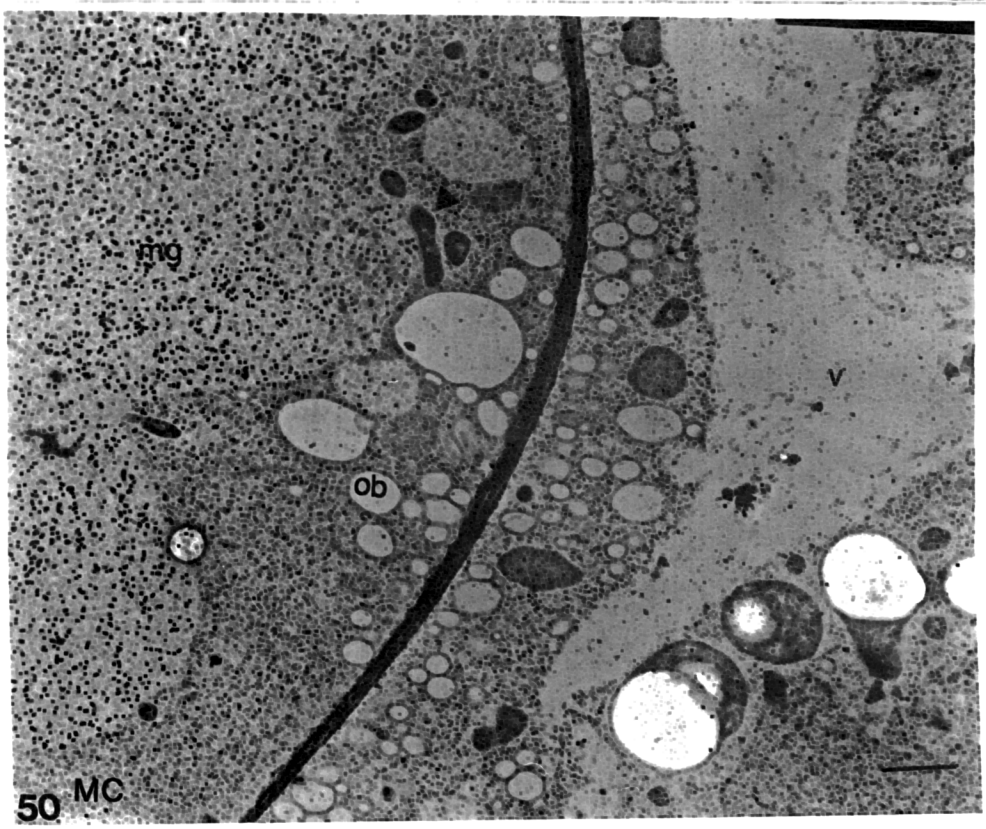
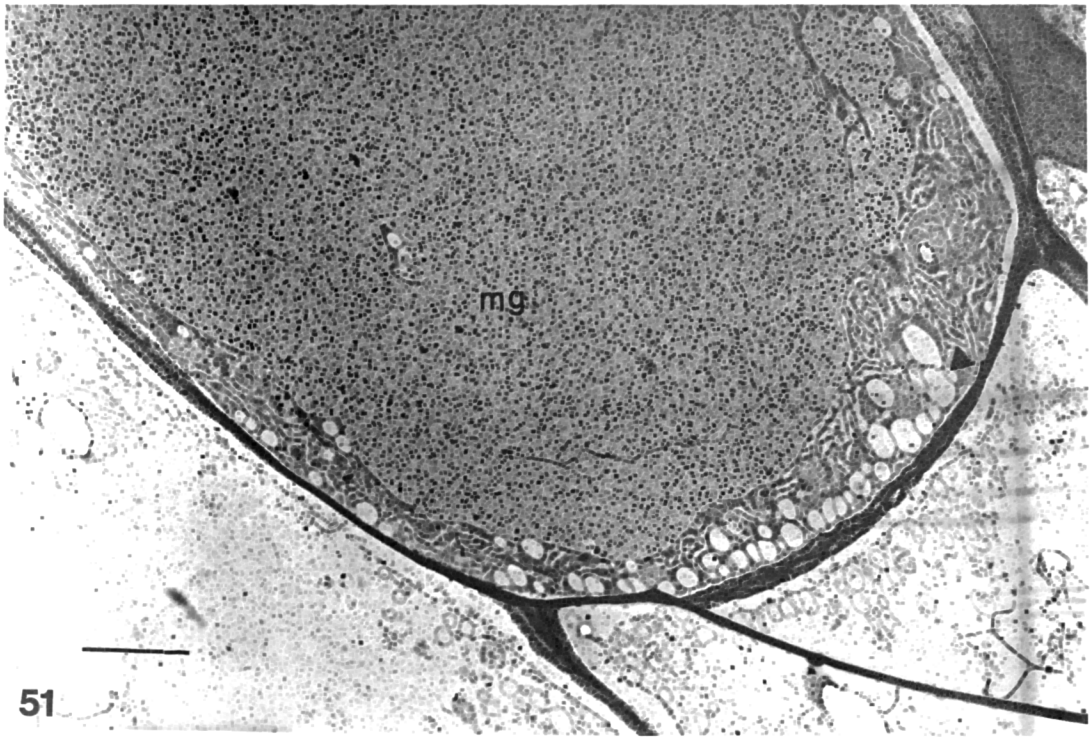


Plate-3.49- Immunogold labelling of myrosinase in a myrosin cell of a "161-inoculated" radicle. Again the localization was restricted to the grains, but the grains appeared to have a less dense matrix. Bar: 2µm.

Plate-3.50- Higher magnification of part of the cell above (Plate-3.49-, ▲). Myrosin cell adjacent to an aleurone cell. The myrosin cell shows a positive reaction in the myrosin grains; the aleurone cell demonstrates a lack of labelling in vacuole or in the cytoplasm. Bar: 2µm.



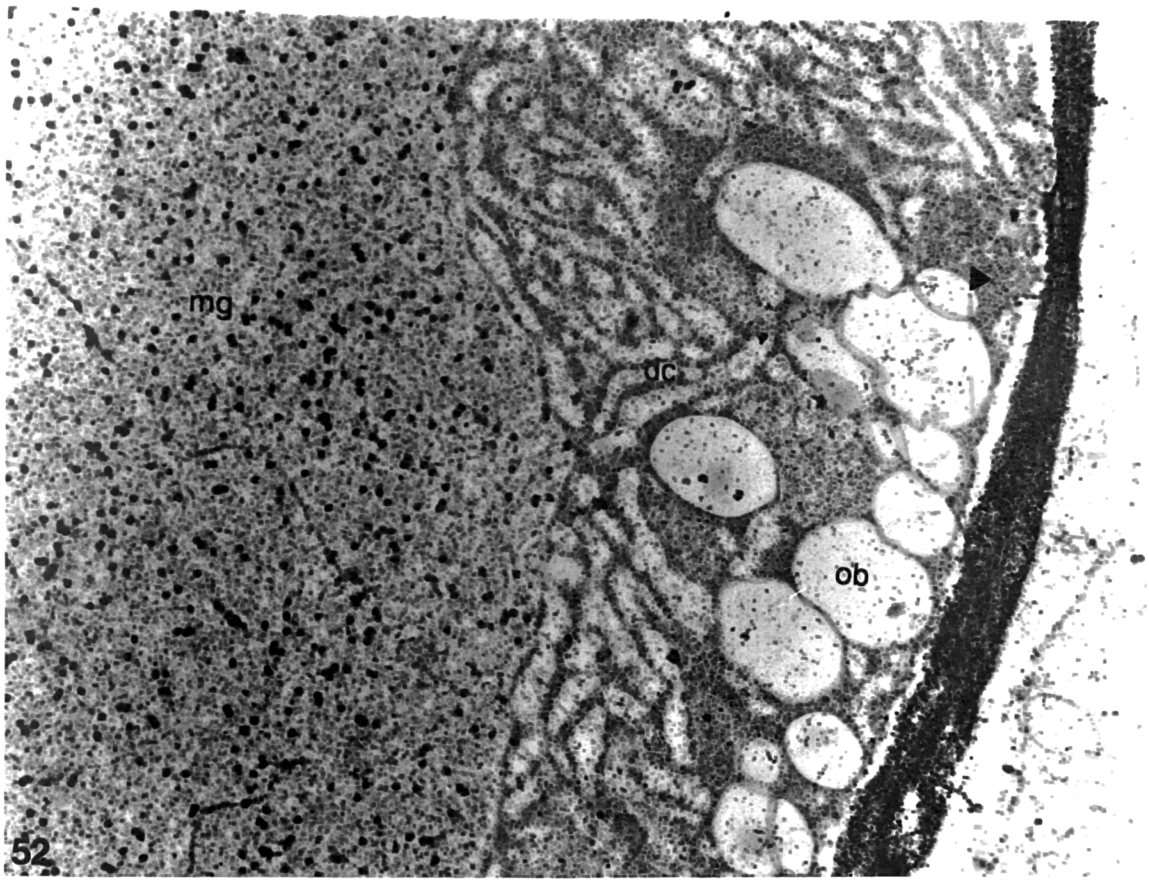
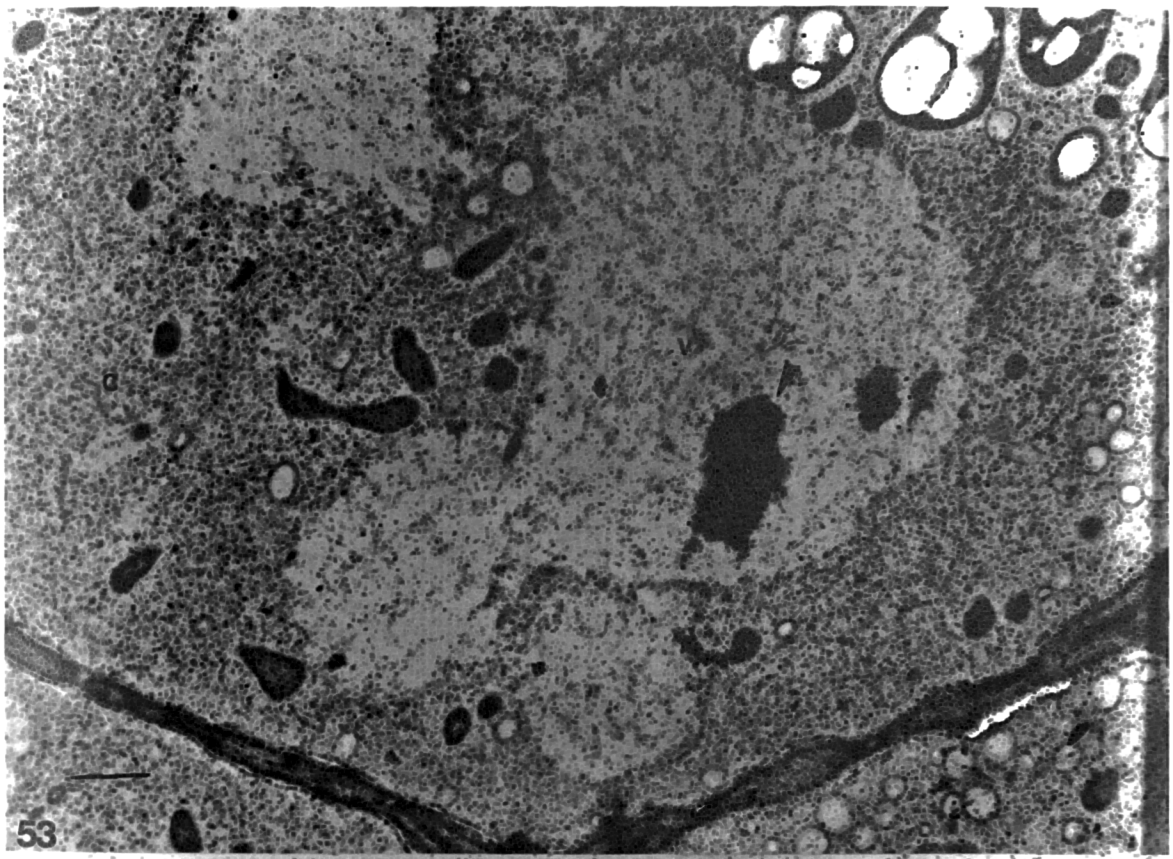


Plate-3.51- Myrosin cell of radicle inoculated with virulent isolate 161. One very large myrosin grain can be seen with positive labelling. Bar: 2µm

Plate-3.52- Higher magnification of part of the above cell (Plate-3.51-, ▲). No labelling was seen in the dilated cisternae (dc) of the ER, plasma membrane (pm), or oil bodies (ob) in the myrosin cell. Bar: 500nm.



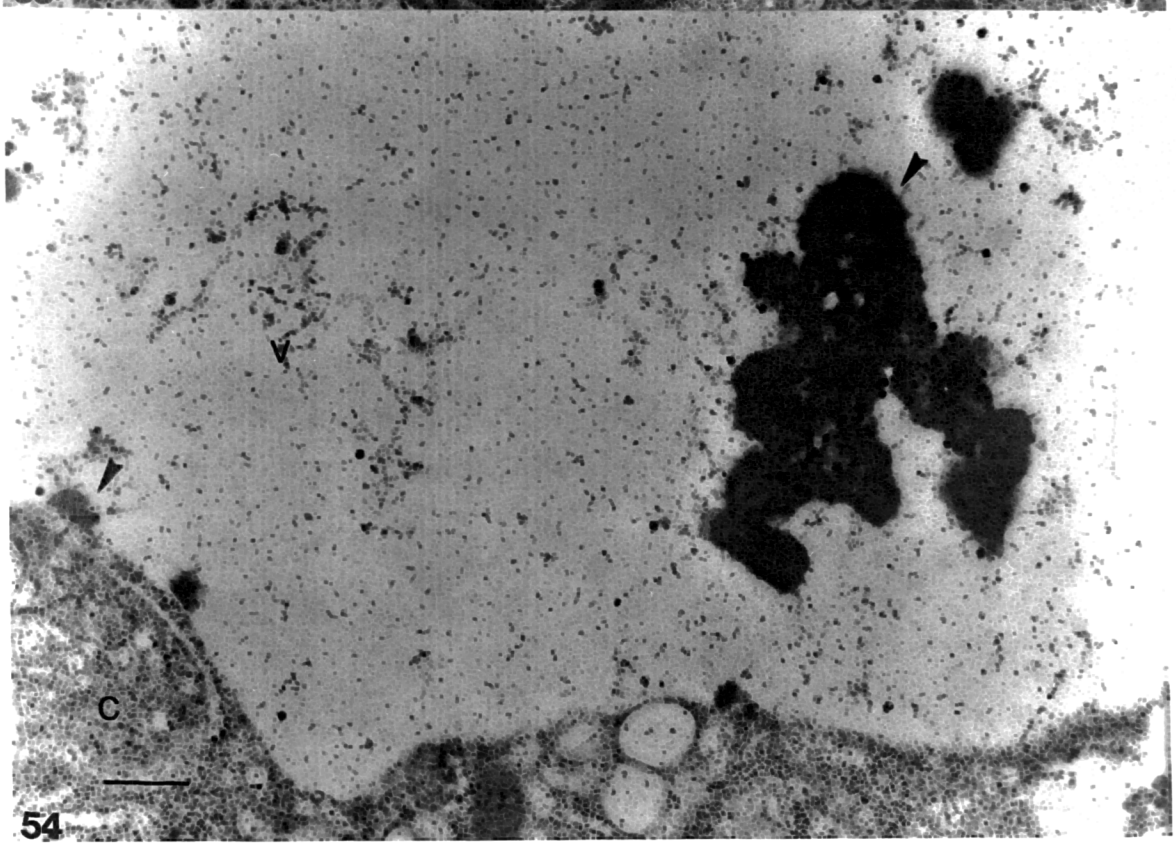
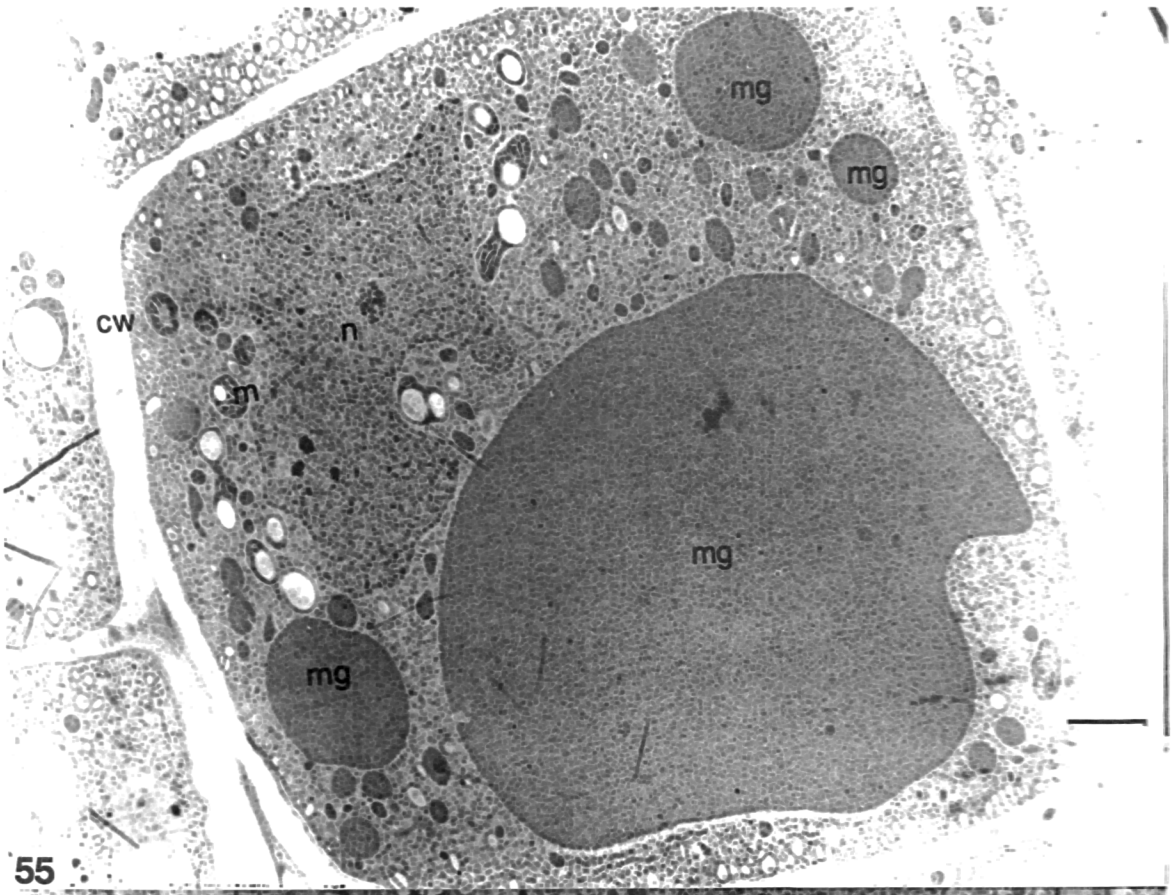


Plate-3.53- In the vacuoles of aleurone cells some amorphous material (arrow head) was observed which was electron-opaque and showed labelling. Bar: 1µm.

Plate-3.54 High magnification of area shown in Plate-3.53-. The amorphous material (arrow head) was always found inside the vacuole, or in the periphery of the vacuole, attached to the tonoplast of aleurone cells. Bar: 500nm.



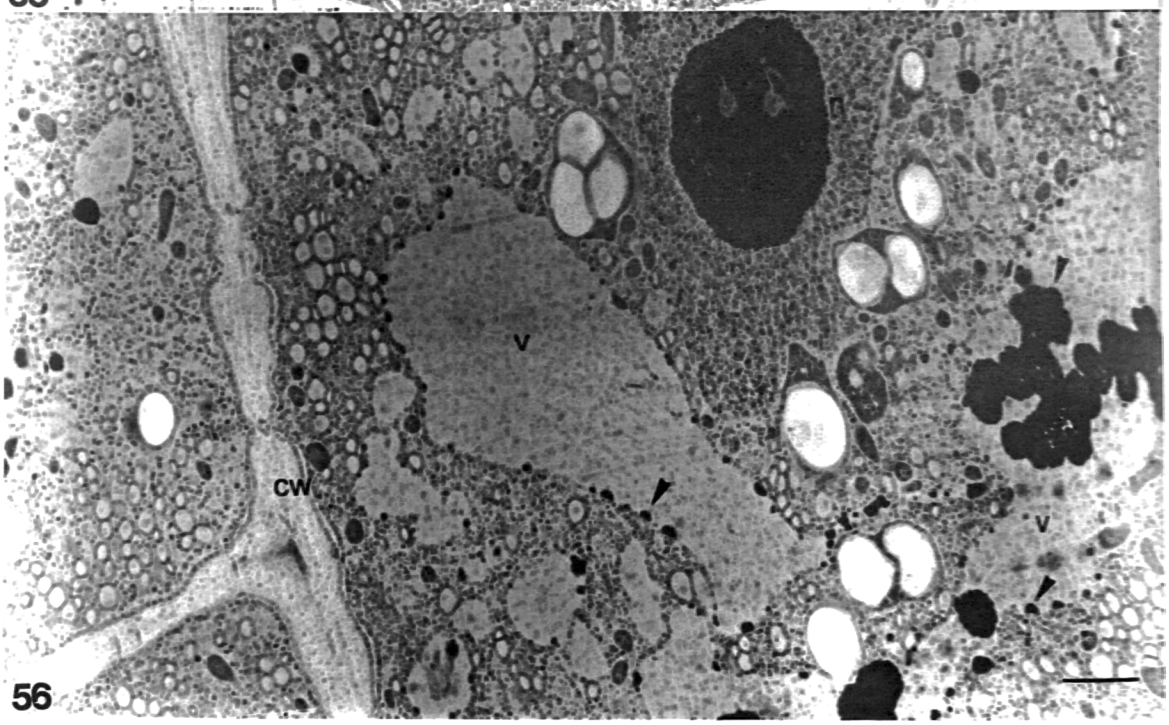


Plate-3.55- Conventional staining for 10 min with 2% aqueous uranyl acetate,, followed by 3 min staining with Reynolds's lead citrate of a myrosin cell in a radicle of oilseed rape. Myrosin grains can be clearly distinguished. Bar: 2µm (n=nucleus, cw=cell wall, m=mitochondria)

Plate-3.56- Conventional staining (see Plate.-3.55-) of an aleurone cell. Vacuoles show some inclusions of amorphous material which were electron-dense. These were present at the periphery, or inside, the vacuole. This was the only other subcellular material which showed specific labelling when immunogold labelling was performed. Bar: 2µm. (v=vacuole).

3.2.2.3. Immunogold localization of myrosinase in roots of *Brassica napus* cv. Cobra (in control plant sections and in sections of 130-, 161-inoculated plants).

The root tips (removed at 1mm from the root tip) of control and plants inoculated with either a virulent isolate 161 of the pathogenic diploid strain viz. *V.d.longisporum* or an avirulent isolate 130 of a non-pathogenic strain (tomato) of *V.dahliae* were prepared for electron microscopic studies (conventional and immunocytological staining) as described in section 2.5.2.2.2 (fixation -a-, and immunogold labelling procedure-1-, page 108). Seedlings of oilseed rape cv. Cobra were inoculated by the root-dipping method when they were 2 week-old and prepared for EM at 3, 10 and 15 days post-inoculation. Figure-3.35- gives a schematic map of the various zones of the extreme root tip of *Brassica napus var. oleifera* cv. Cobra.

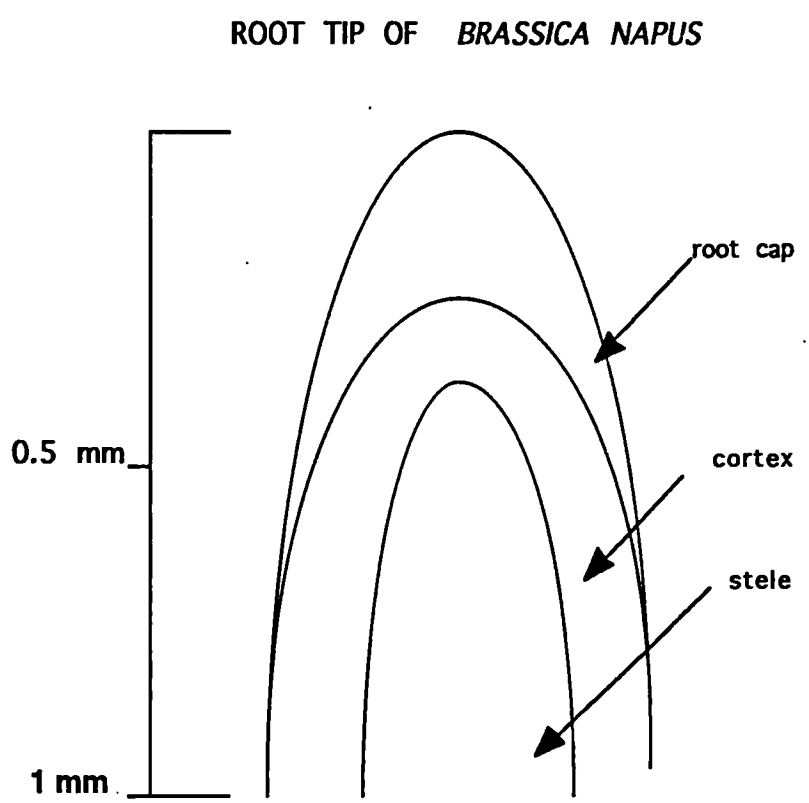


Figure-3.35- Schematic map of root tip of *Brassica napus.*

In control sections of root tips of oilseed rape, colloidal gold labelled myrosinase was only associated with **electron opaque areas and bodies in vacuoles** of the root cap cells and the outer most cortical area (Plate-3.57-). No such myrosinase accumulating bodies (mab) were found in the parenchyma cells of the stele, or the epidermal cells and at the very end of the root tip. Meristematic cells (undifferentiated) with very small vacuoles in the meristematic tip and central cylinder also lacked the enzyme (Plate-3.65-).

Subcellularly, the enzyme was always found in the vacuole. No labelling was found in the cytoplasm (c), or the nuclei (n), or in cell walls (cw) or any cytoplasmic membrane. The enzyme in the vacuoles was always associated with electron opaque areas which were sparse (less gold labelling) or with an homogenous granular material which formed almost spherical bodies, ie. mab (Plates-3.58,-3.59,-3.60,-

3.61-). Small vacuoles of the root cortical cells contained from 1-2 spherical bodies. The most labelling was found in the first layer of the cortical cells (Plates-3.57-, 3.66-), immediately underneath the epidermal cells. In these cells, the one large vacuole dominated the interior of the cell which was full of enzyme in these electron opaque areas; also a number of small mab were formed. Other cells were contained less enzyme (only the first 3-4 cortical layers contained the enzyme). The inner cells of the cortex with the very large vacuoles (Plates-3.62,-3.66- ,-3.67-) and these of the stele lacked these electron-opaque areas and the enzyme. Thus cells that lacked electron-opacity areas in their vacuoles also lacked the enzyme.

Conventional staining of sections of the same tissue revealed the nature of these electron-opaque areas (Plate-3.76-). The material had the same staining properties as the cytoplasm (homogenous granular content) but was located inside the vacuoles and was separated from the rest of the vacuole by a membrane (Plates-3.77-,-3.78-). Cells of the stele and the meristematic zone that lacked the enzyme, had vacuoles which also lacked these bodies.

In roots inoculated with either the virulent isolate 161 of the pathogenic diploid strain viz. *V.d.longisporum* or the avirulent isolate 130 of a non-pathogenic strain (tomato) of *V.dahliae*, no fungal hyphae were observed in the root tip area associated with the enzyme.

Occasionally, in root tips of the '161-inoculated plants', gold labelling was found in the intercellular spaces of the 4-5 layers of the root cortex (Plate-3.71-). The same phenomenon was also observed to a lesser extent in control uninoculated plants (Plate-3.70-). Generally, the enzyme in sections of roots inoculated with either the virulent isolate 161, or the avirulent isolate 130, was also found in the vacuoles (Plate-3.66-) being associated with particulate bodies (Plate-3.67-) and electron-opaque areas, as in the control root tips. More myrosinase accumulated bodies seemed to appear in the material inoculated with either the virulent isolate 161, or the avirulent isolate 130, but this was not certain because of the difficulty in associating the enzyme with the localization of the fungus in this tissue. A number of sections of the root tip were observed under the light microscope (toluidine blue stained sections), but it was not possible to localize either 161 or 130 hyphae in this particular part of the tissue at 3, 10 and 15 days post-inoculation.

Plate-3.57- Immunogold labelling of myrosinase in cortical cell of root tip of control plants of *Brassica napus* cv. Cobra. Epidermal cell (ec) shows lack of colloidal gold-labelled myrosinase. The first cortical cell under the epidermis is dominated by a very large vacuole (v), with electron opaque spherical bodies (arrows) where the majority of gold particles is seen. No colloidal labelled myrosinase is seen in cell wall (cw), plasmalemma, tonoplast or cytoplasm (c). The myrosinase is confined in the vacuole. The grid supporting the tissue was incubated in primary antibody M1 with procedure 1 and was observed with an Hitachi H-7000 electron microscope at 75V. (Magnification X 10K).

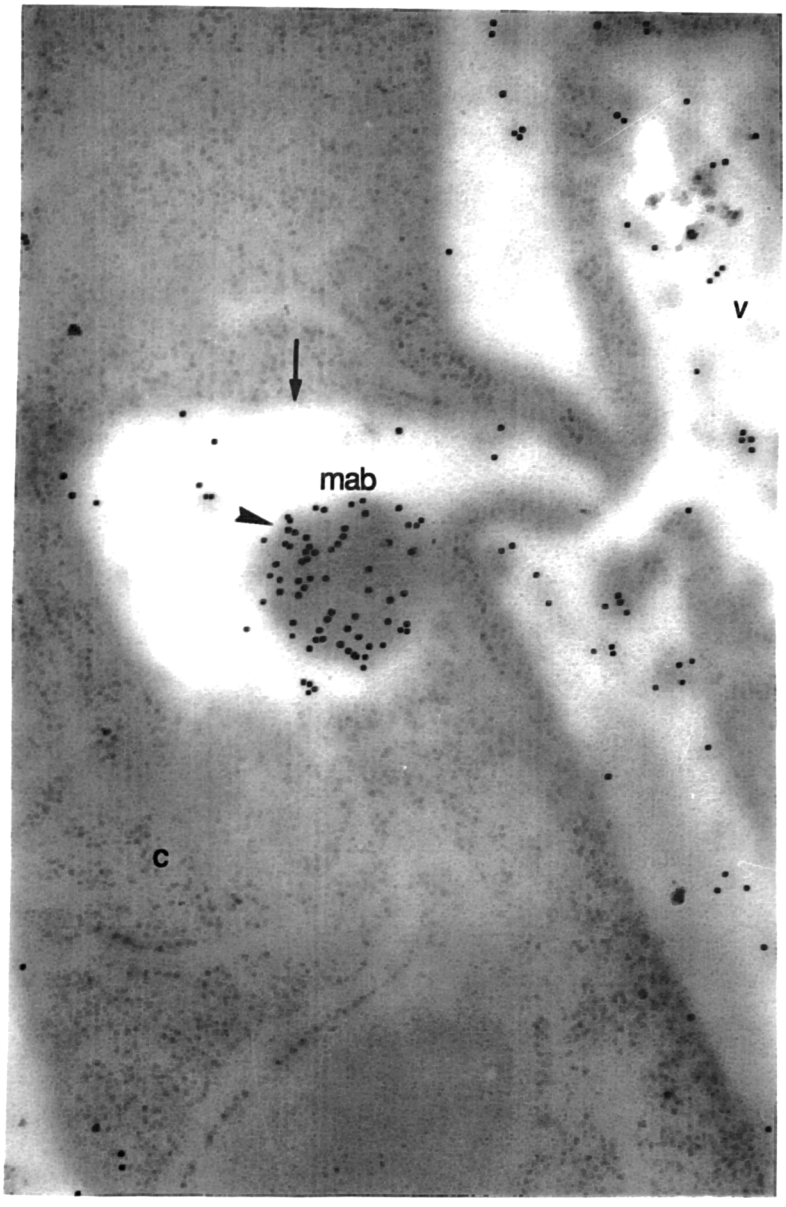


Plate-3.58- Detail of one of the above (Plate-3.57-) myrosinase-accumulated bodies (mab) in vacuole of a cortex cell of the root tip. Specific labelling on this almost spherical electron opaque area can be seen. (Magnification X60K).

Plate-3.59- Two myrosinase accumulated bodies (mab) in vacuole of a cortical cell (3d layer under the epidermis) in control root tip of oilseed rape. (Magnification X60K).

Plate-3.60- One myrosinase accumulated body in the vacuole of a cortical cell (2d layer under the epidermis) in control root tip of oilseed rape. The vacuome of this cell consists of a number of small vacuoles. Only vacuoles with electron opaque, spherical bodies show a positive reaction to the labelled antibody. Some other small vacuoles can be seen which lack myrosinase accumulated bodies. (mag X30).

Plate-3.61- One myrosinase accumulated body (mab) in the first cortical cell under the epidermis. This particulate myrosinase accumulated body occupies the whole of the small vacuole. (Magnification X60K).

Plate-3.62- Empty vacuoles (v) lacking mab in the 4th cortical cell layer under the epidermis of control, root tip of oilseed rape (Magnification X7k). No labelling can be seen in the nucleus (n) or cytoplasm (c) or plasma membrane (pm).

Plate-3.63- Detail of the above (Plate-3.62--) vacuole showing lack of labelling. No electron opaque areas can be seen in the vacuoles (as was observed in vacuoles of cortical cells of the first, second and third layer under the epidermis).

Plate-3.64- Empty vacuoles (v) of myrosinase accumulated bodies in the 5th cortical layer under the epidermis in control, cortical root tip cells of oilseed rape (Magnification X18K).

Plate-3.65- Meristematic undifferentiated cells of control root tip with clusters of small vacuoles which lack the enzyme myrosinase. (Magnification X6K). One mab, (arrow) can be seen in a cell that has started to differentiate (much larger vacuoles).

Plate-3.66- Immunogold labelling of myrosinase in cortical cell of '161 inoculated' root tip of *Brassica napus* cv. Cobra. Epidermal cell (ec) showing lack of colloidal gold labelled myrosinase. Vacuoles in the first cortical cell layer under the epidermis contain electron opaque spherical bodies (arrows), where the majority of gold particles is seen. No colloidal labelled myrosinase is seen in cell wall (cw), plasmalemma, tonoplast or cytoplasm (c). The myrosinase is confined to the mab and in electron opaque areas inside the vacuole. (Magnification X5K).

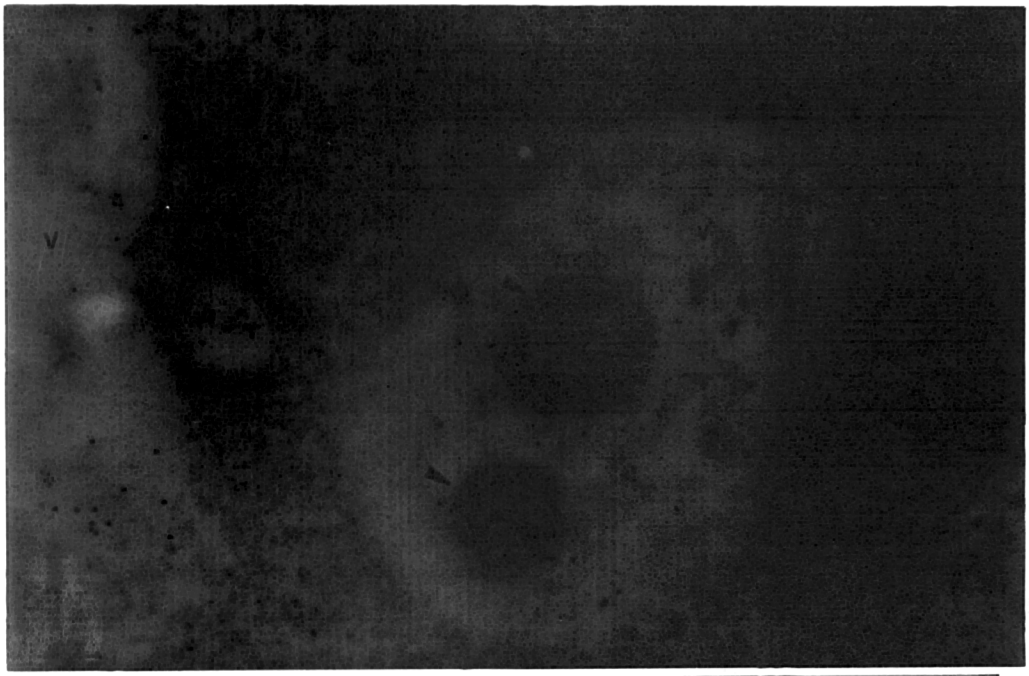
Plate-3.67- Detail of one vacuole (v) of the above cortical cell (Plate-3.66-). Myrosinase accumulated body (mab) with specific labelling can be seen. Also a positive reaction to a lesser extent can be seen in electron opaque areas with no particulate shape, inside the vacuole. (Magnification X34K).

Plate-3.68- Empty vacuoles with no labelling of cortical cells in the 5th layer of the epidermis of '161 inoculated' root tip of oilseed rape. There is no label in any organelle, or cytoplasm. Inside the vacuole, an electron dense area can be seen with no label. (Magnification X14K).

Plate-3.69- Empty vacuole of a cortical cell in the 5th cortical cell layer cell from the epidermis of '130 inoculated' root tip of oilseed rape. This cell did not show any label in any subcellular organelle or in the cytoplasm. (Magnification X20K).

Plate-3.70- Immunogold labelling in intercellular space of two cells (5th-6th) layer in cortex of control root tip of oilseed rape. (Magnification X60K).

Plate-3.71- Immunogold labelling in intercellular space of two cells (5th-6th layer) in the cortex of the '161 inoculated' root tip. (Magnification 30K).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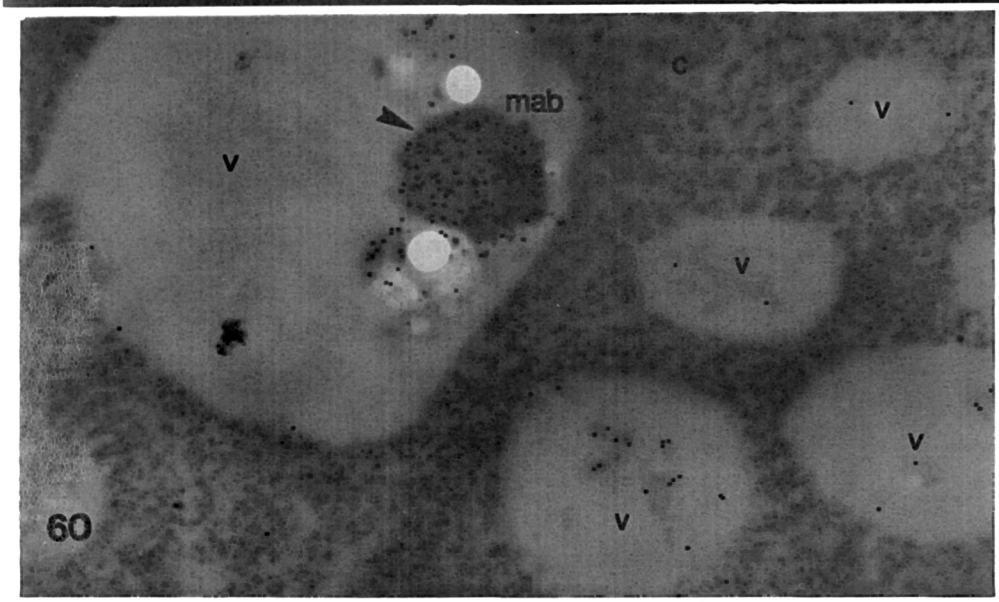
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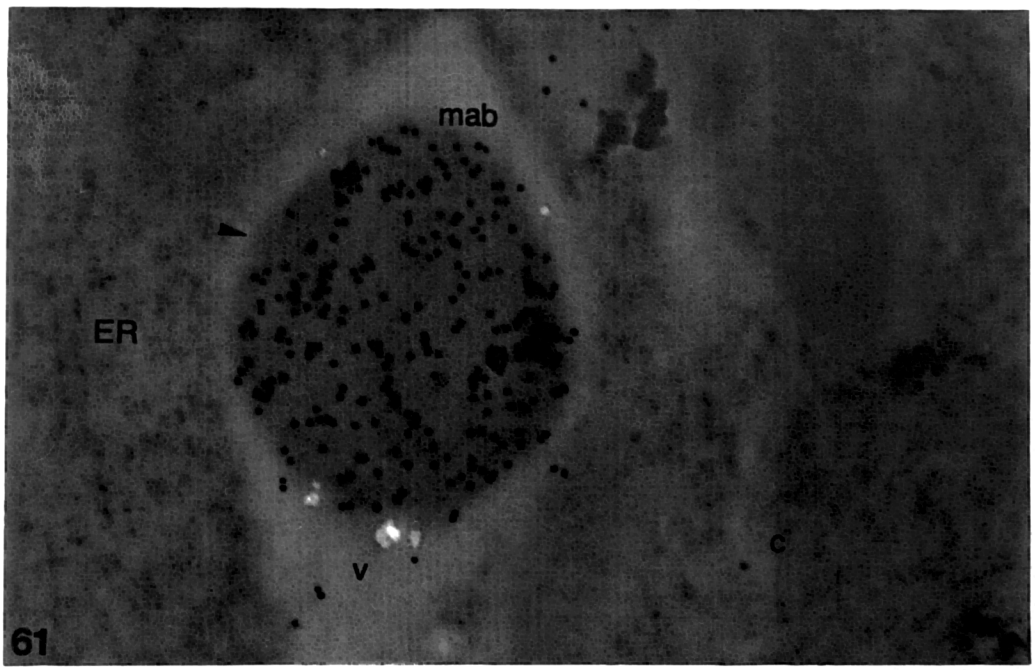
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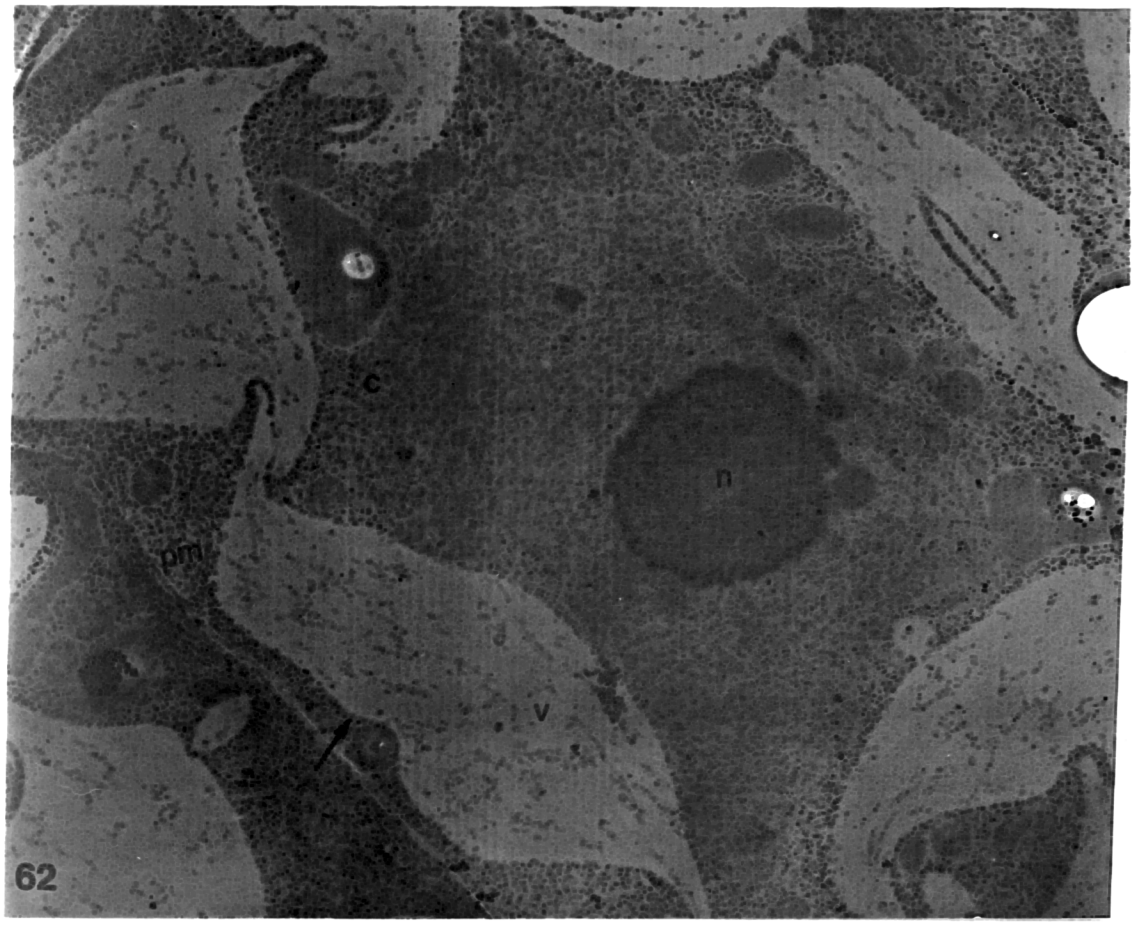

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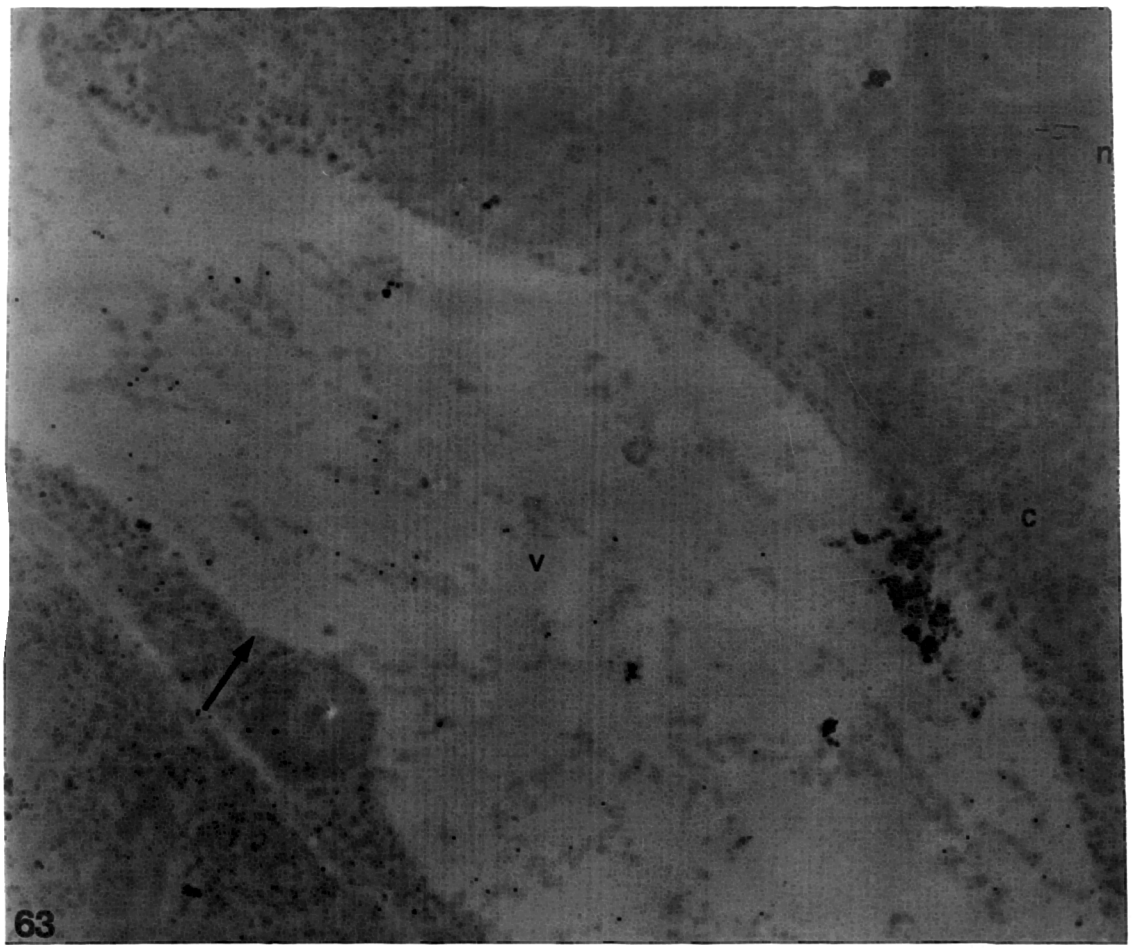

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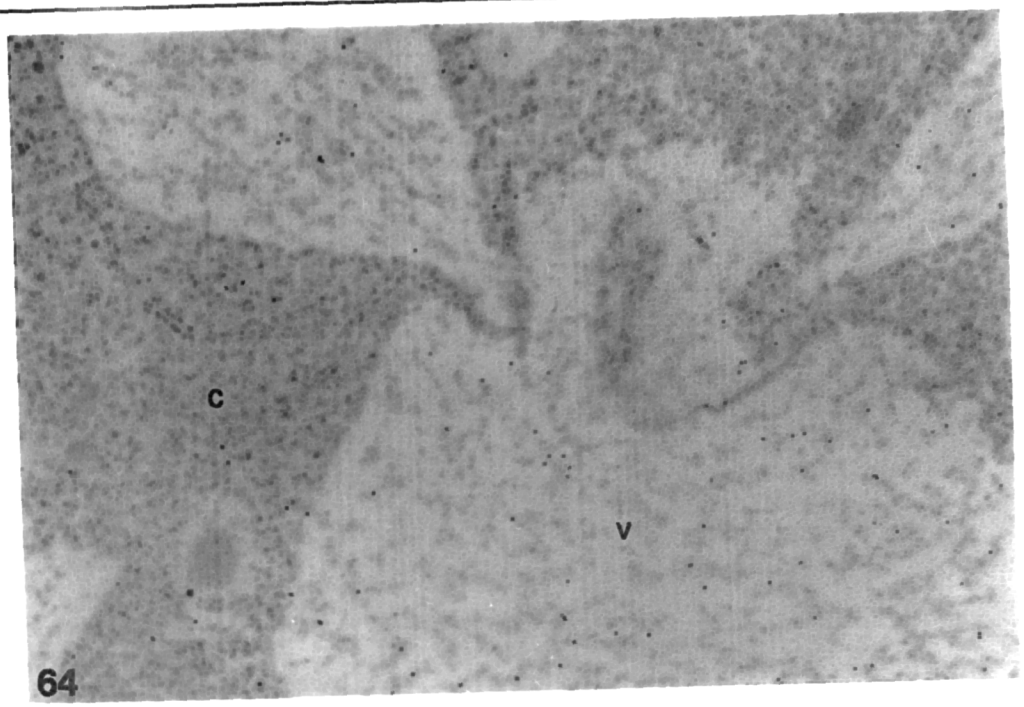

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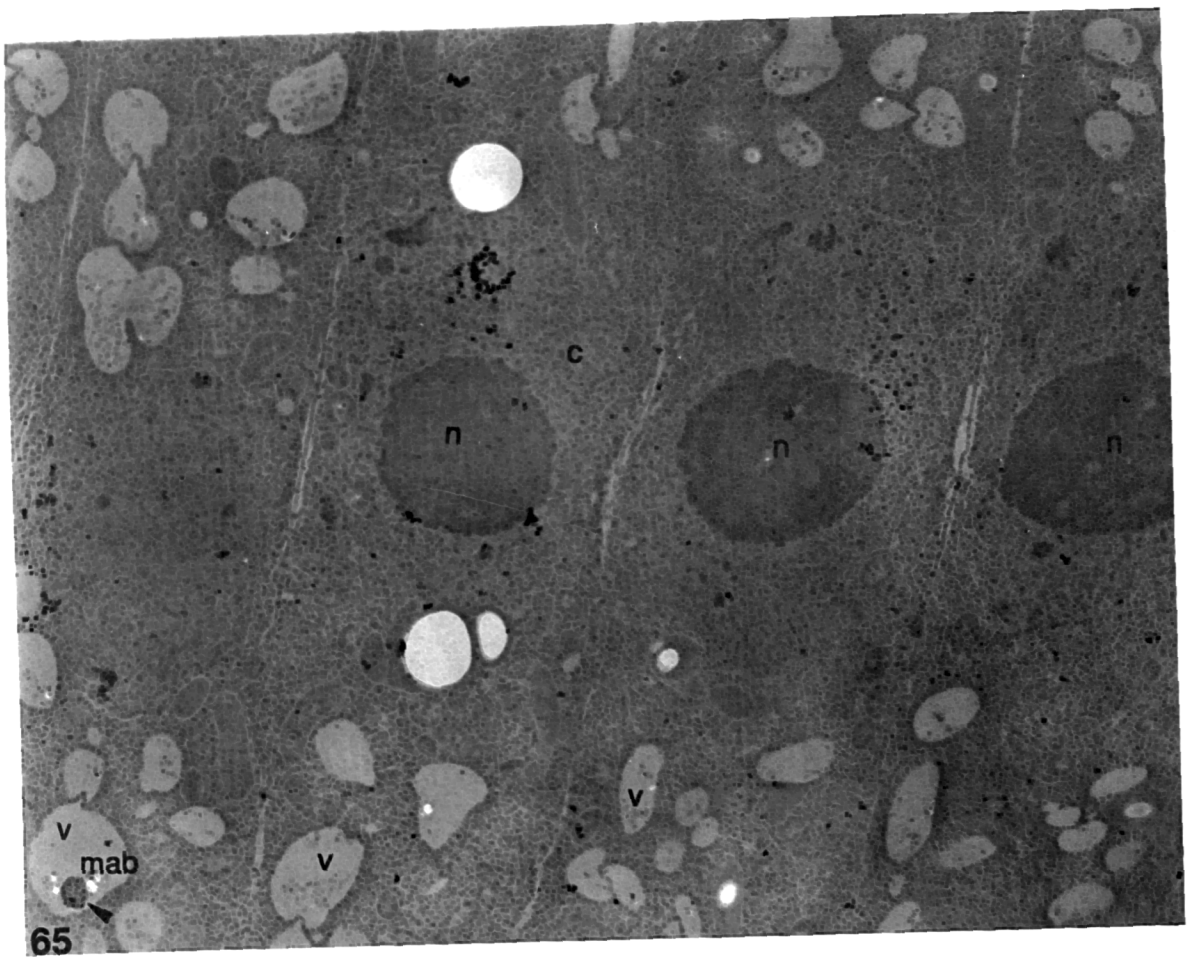

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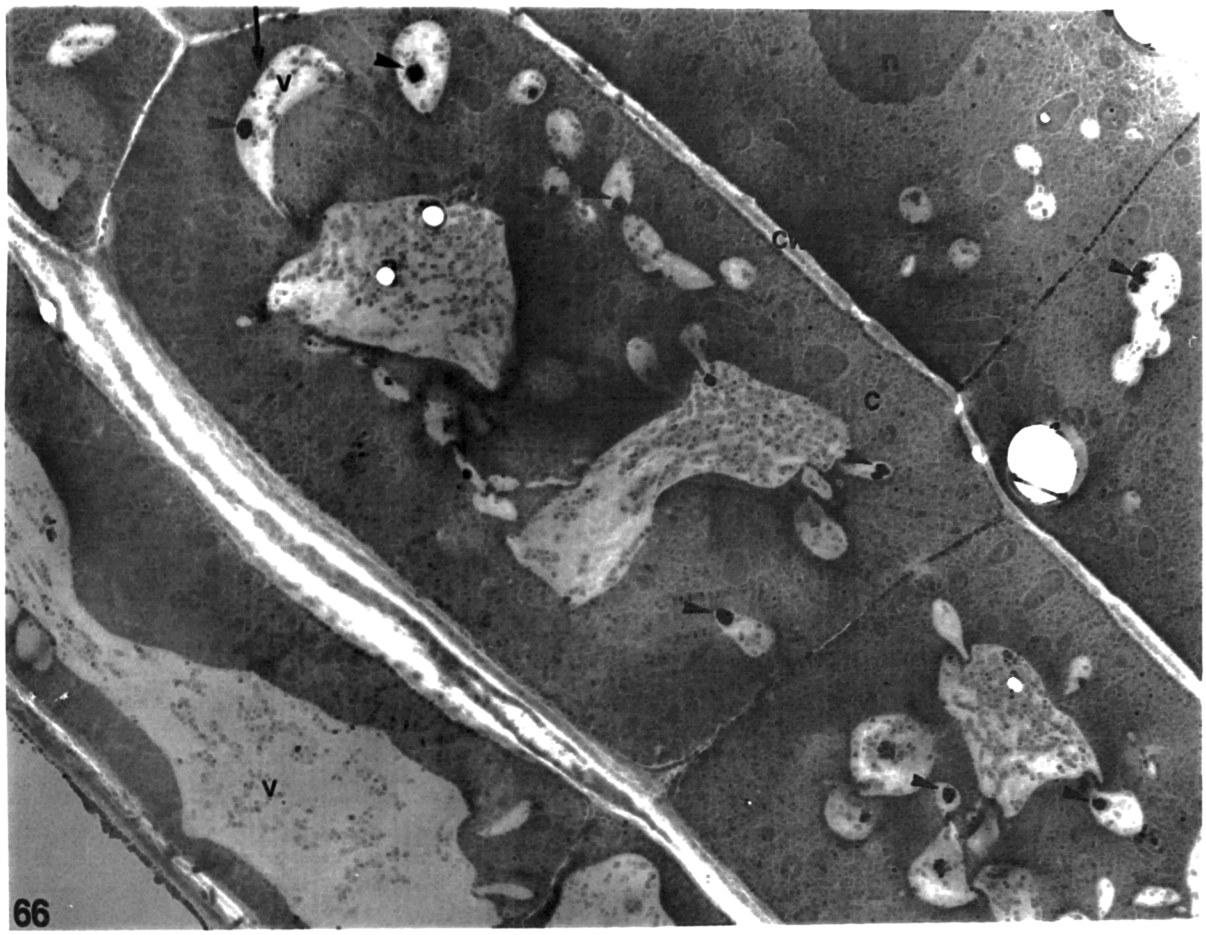

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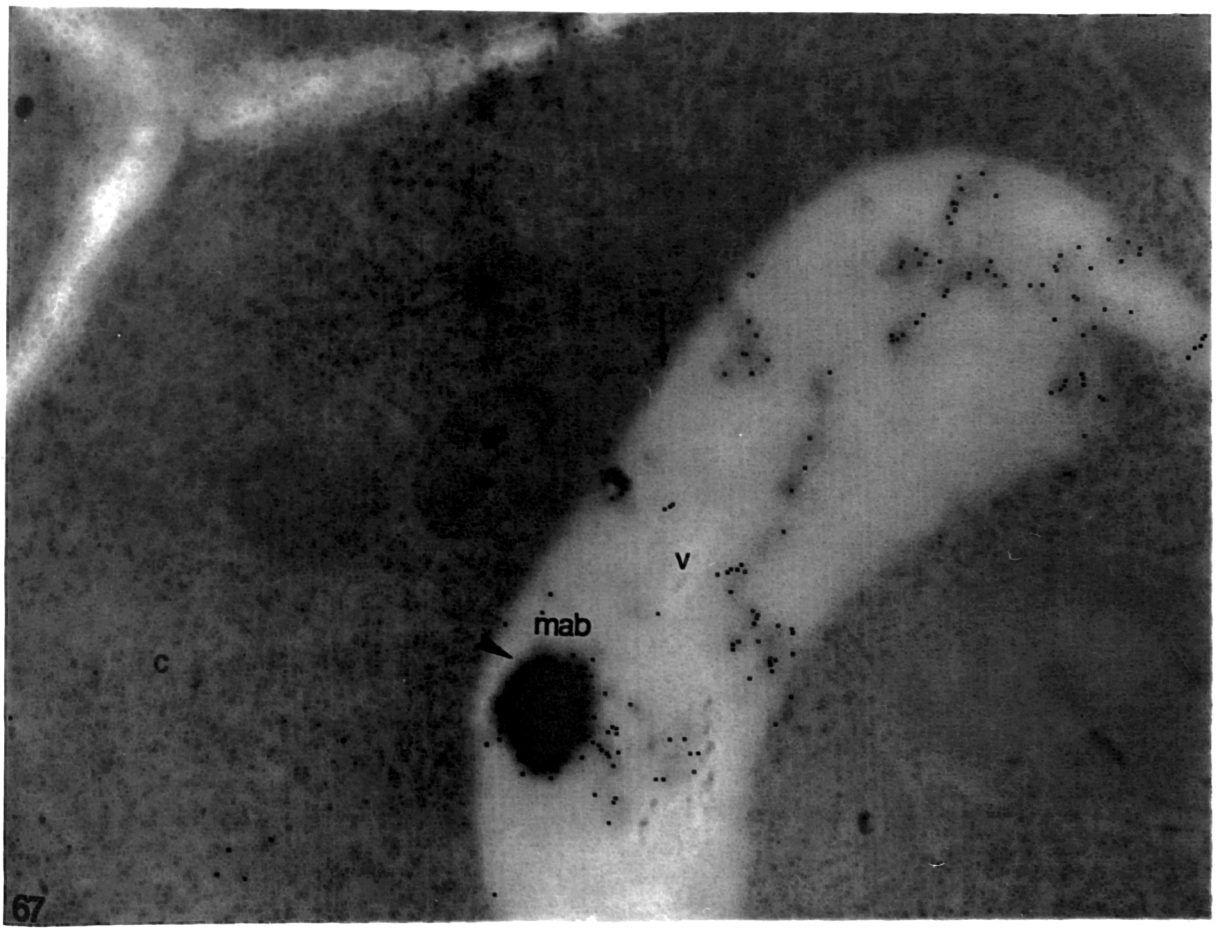

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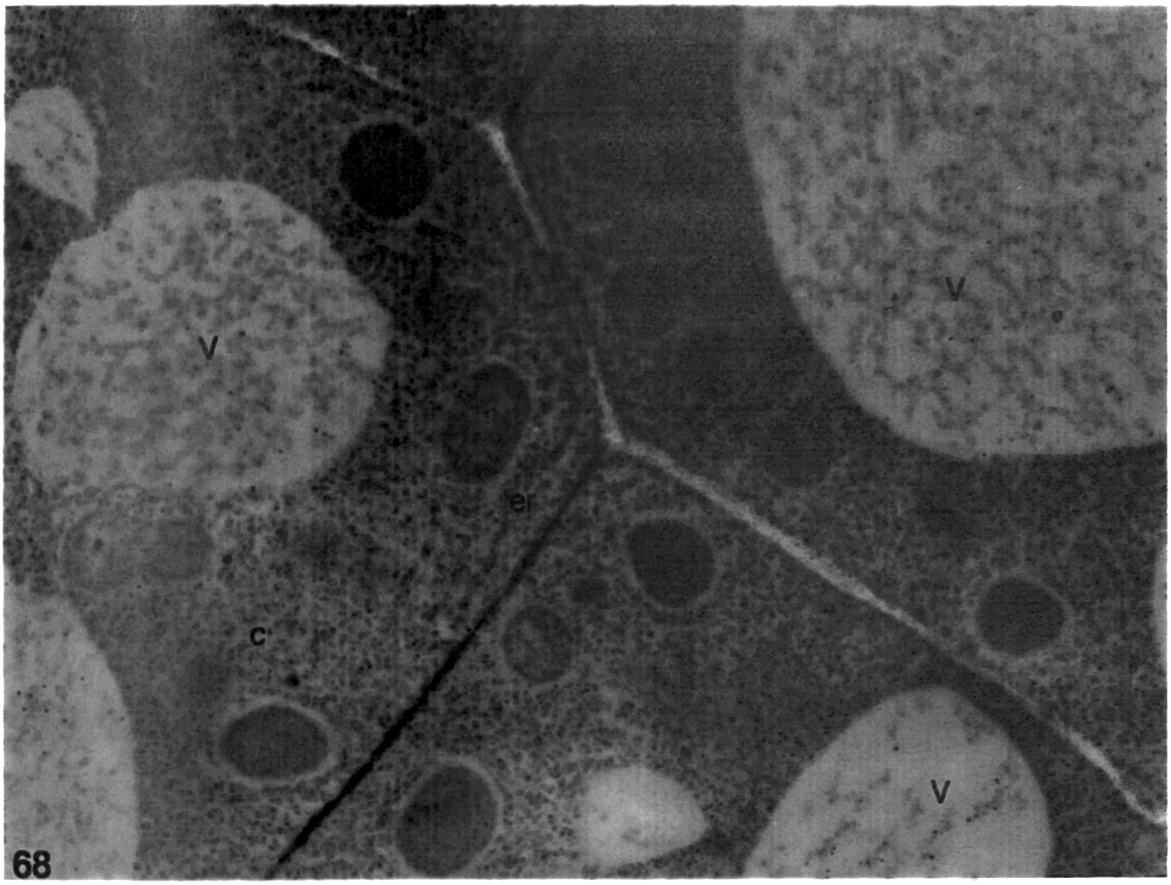

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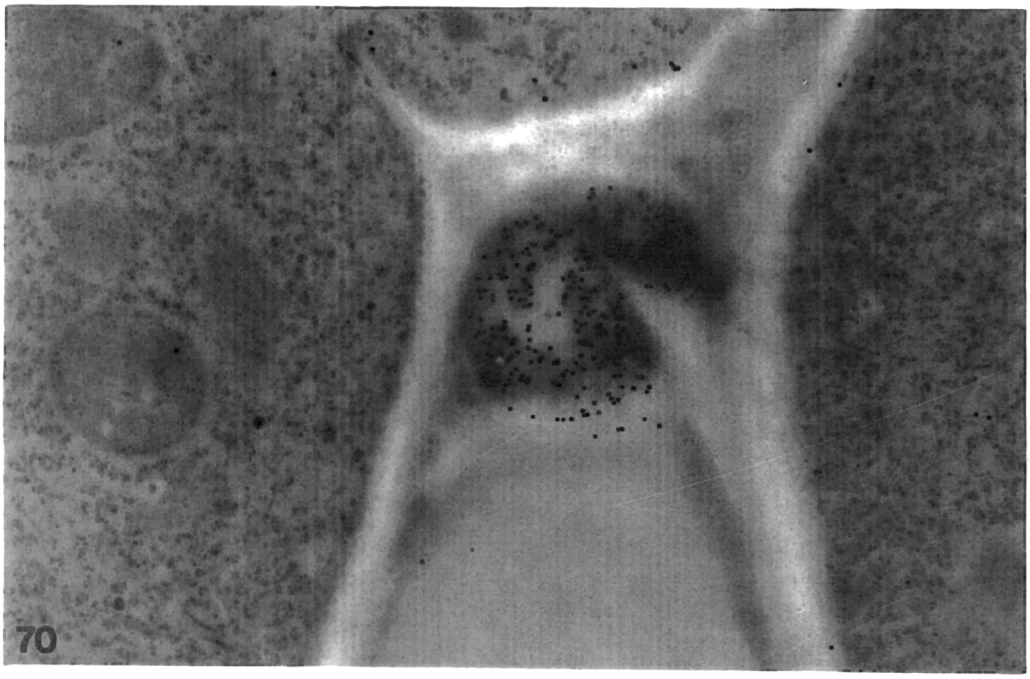
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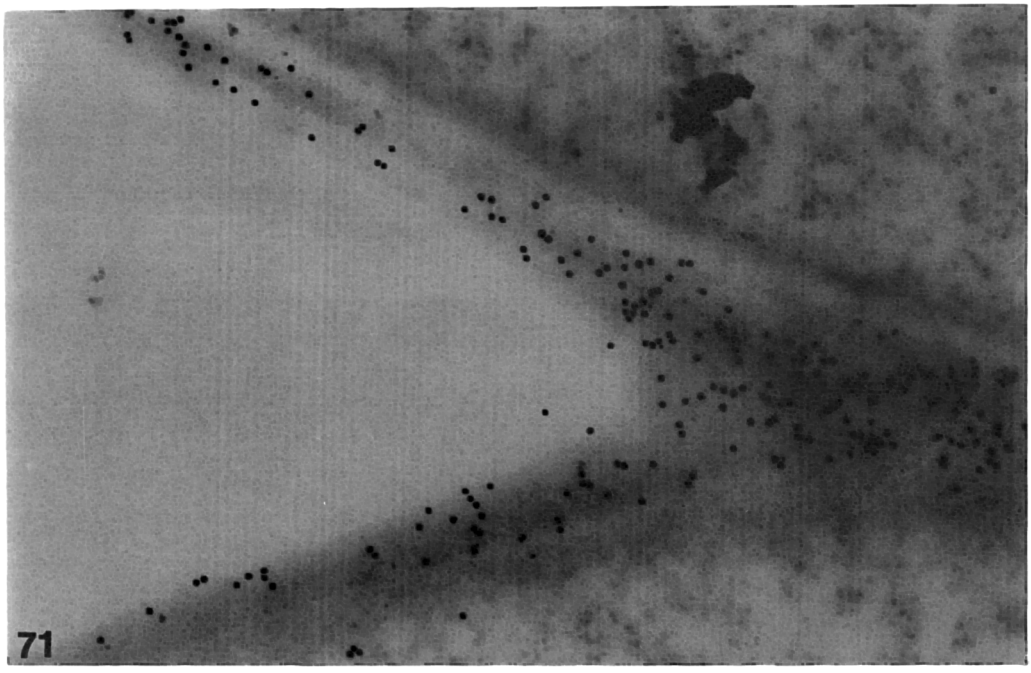
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Plate-3.72- Conventional staining of undifferentiated meristematic cells of the root tip of control plant of oilseed rape. The vacuome of the cells consists of a number of small vacuoles which lack of electron opaque areas. (Magnification X8K).

Plate-3.73- Conventional staining of control cells of the cortex of the first layer underneath the epidermal cells. Electron opaque bodies can be seen inside the vacuole (arrows) where the myrosinase was localized when immunogold labelling was performed. (Magnification X 7K).

Plate-3.74 Conventional staining of cortex cells in the 4th layer underneath the epidermis of '161 inoculated root tip' of oilseed rape. No electron opaque bodies can be seen inside the vacuoles. (Magnification X10K).

Plate-3.75- Conventional staining of a cell at the extreme tip of a '161 inoculated root tip' showing an empty vacuole. (Magnification X30K).

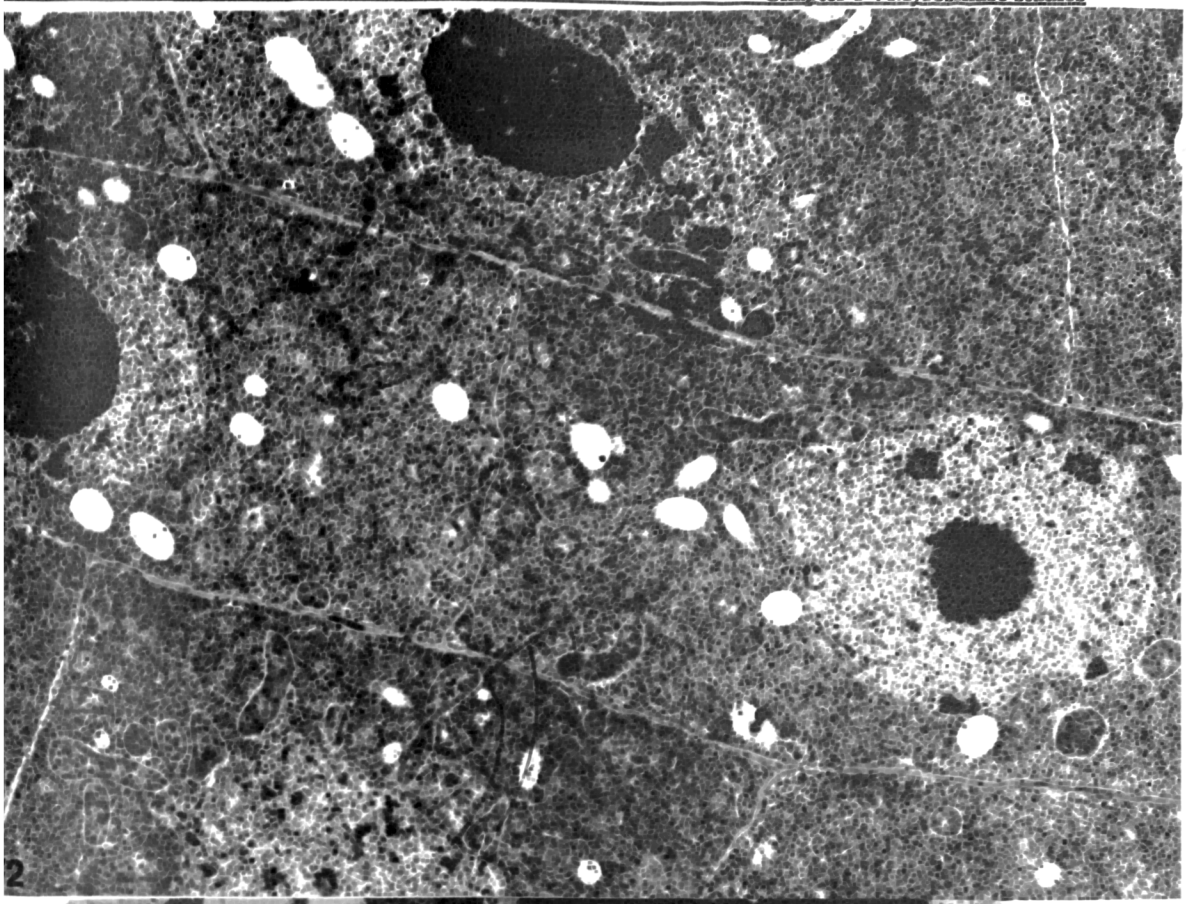
Plate-3.76- Conventional staining of a cortical cell in '161 inoculated' root tip of oilseed rape. A spherical electron opaque body (mab) can be seen inside the vacuole, with the same staining properties as the cytoplasm. A membrane can also be observed (arrow) which separates this body from the vacuole. (Magnification X30K).

Plate-3.77- Detail of the above vacuole (**Plate-3.76-**) with the myrosinase accumulated body. A membrane separates this body from the vacuole sap. The tonoplast (t) can also be seen. (Magnification X40K).

Plate-3.78- Higher magnification of the mab in plate-3.77- which shows a granular appearance similar to the cytoplasm. (Magnification X 120K).

Plate-3.79- Conventional staining of a cortical cell in the second layer underneath the epidermis in control root tip. Two mab can be seen inside the vacuole. The tonoplast can be easily distinguished which separates the vacuole sap from the cytoplasm. (Magnification X 60K).

Plate-3.80- Conventional staining of a cortical root cell in the first layer underneath the epidermis in '130 inoculated' root tip. This section is through the membrane that separates the mab and a net of membranes can be seen to accommodate the mab. (Magnification X60K).

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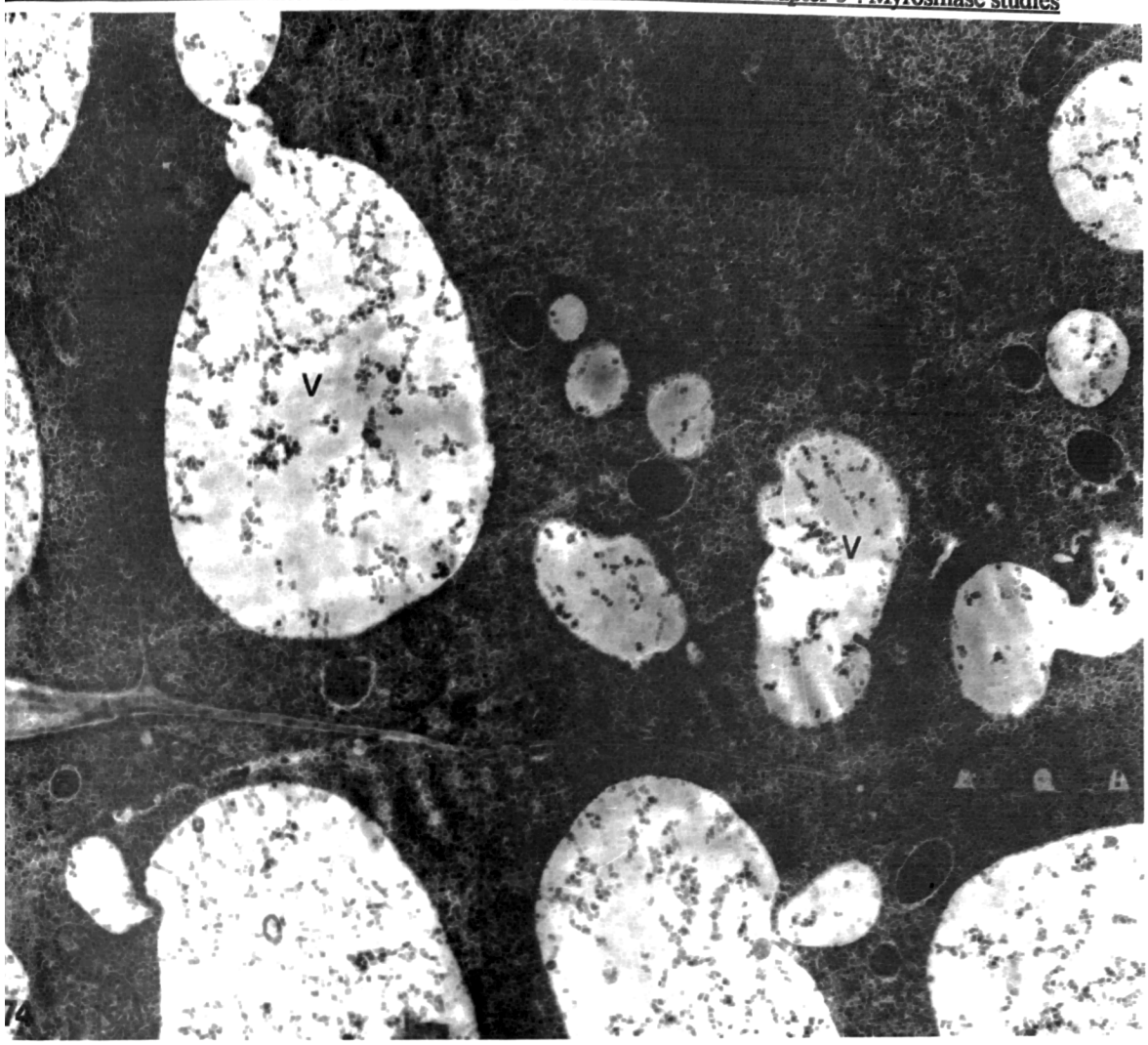

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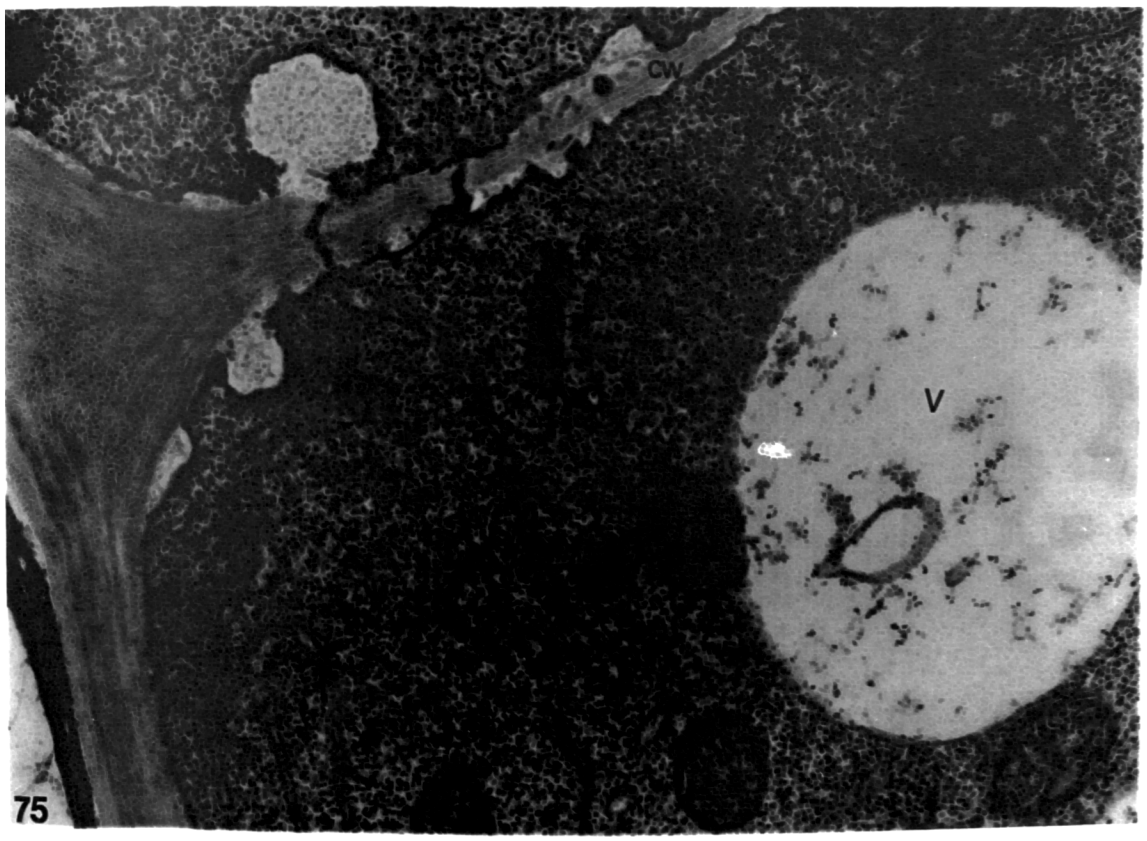

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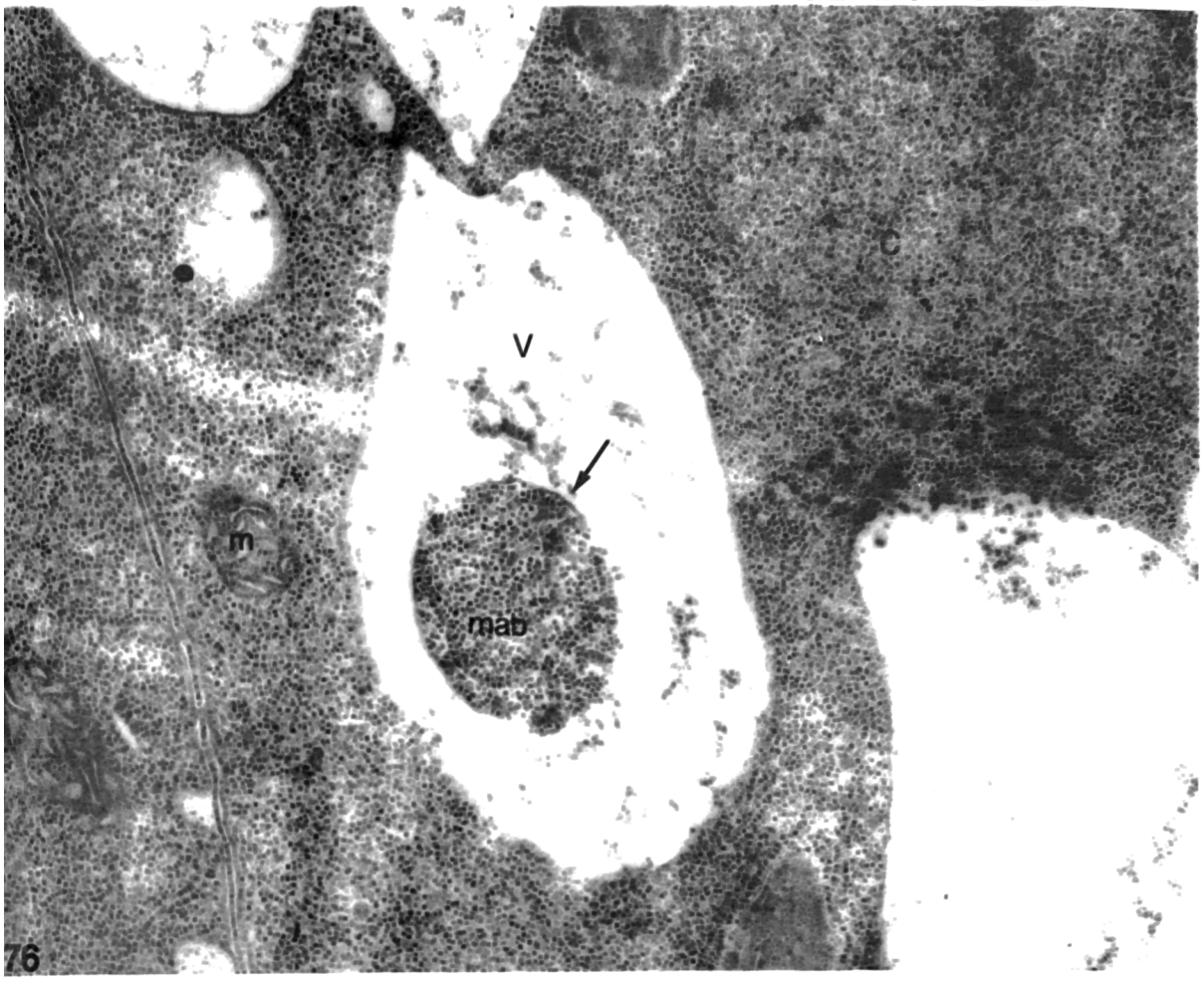

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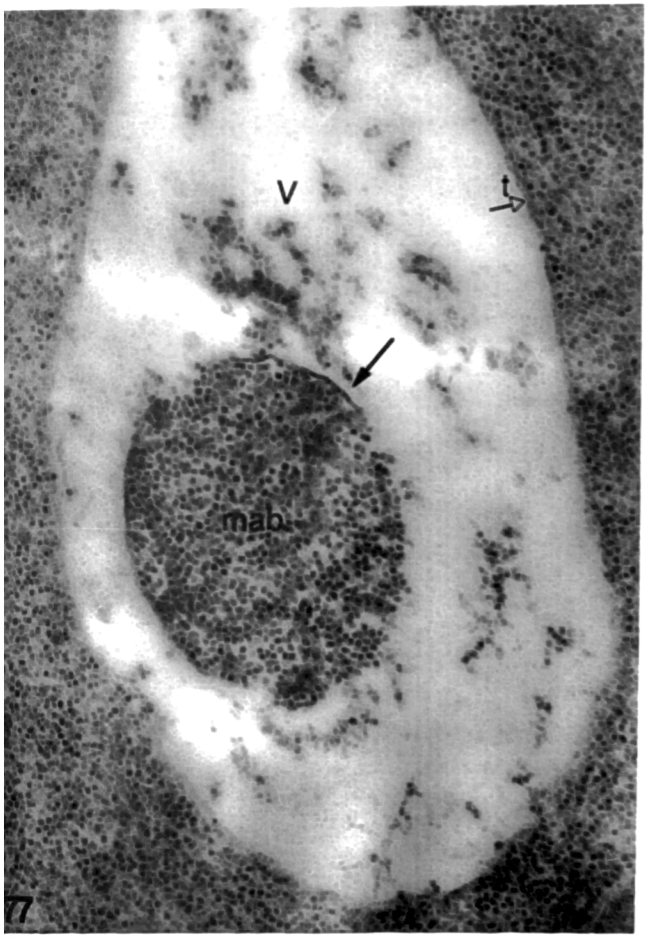

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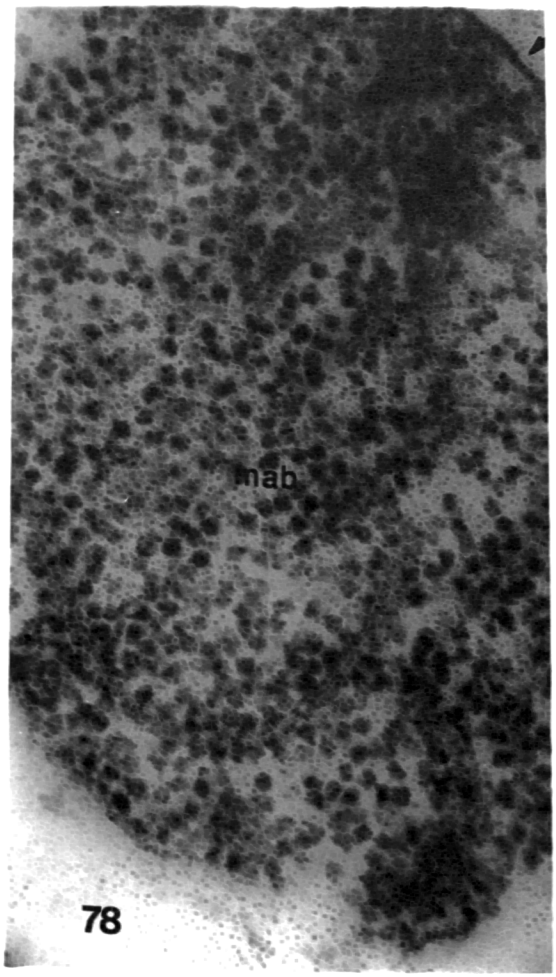

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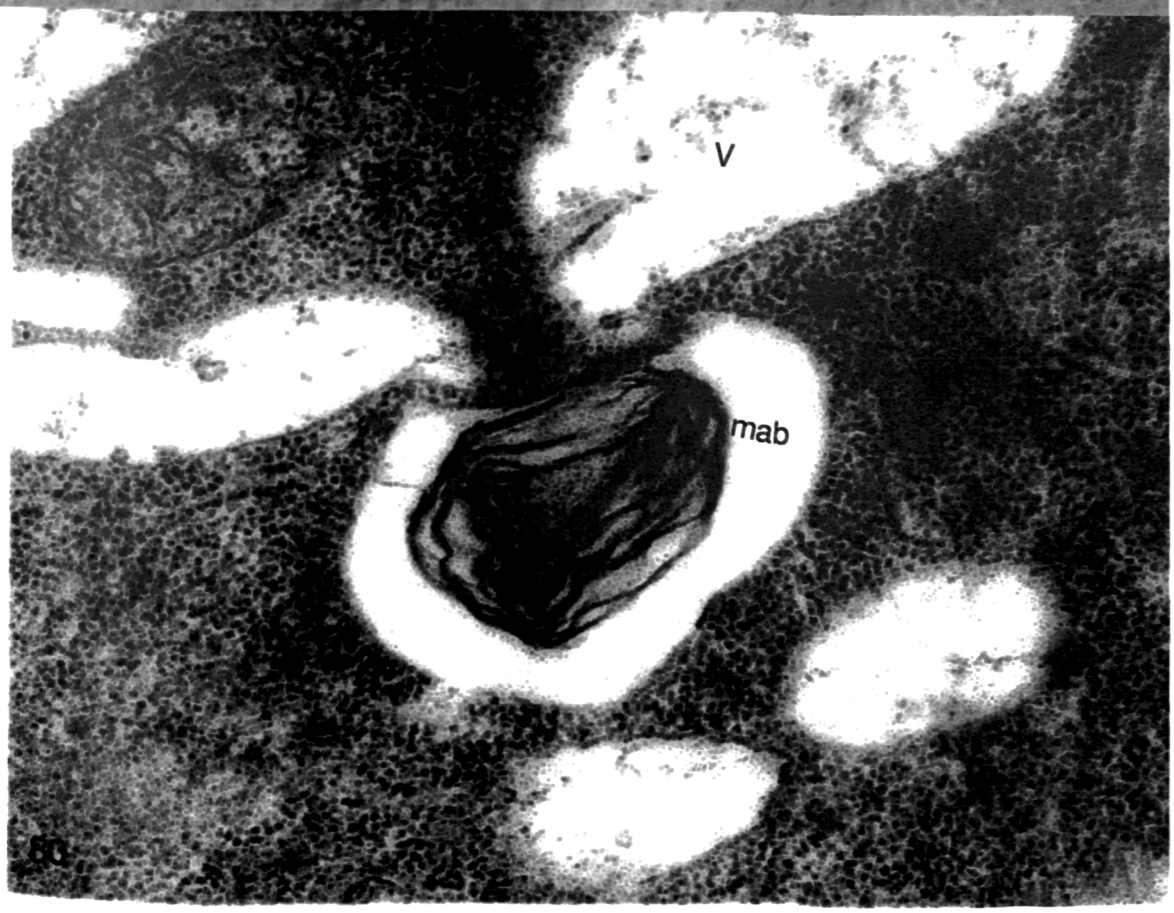

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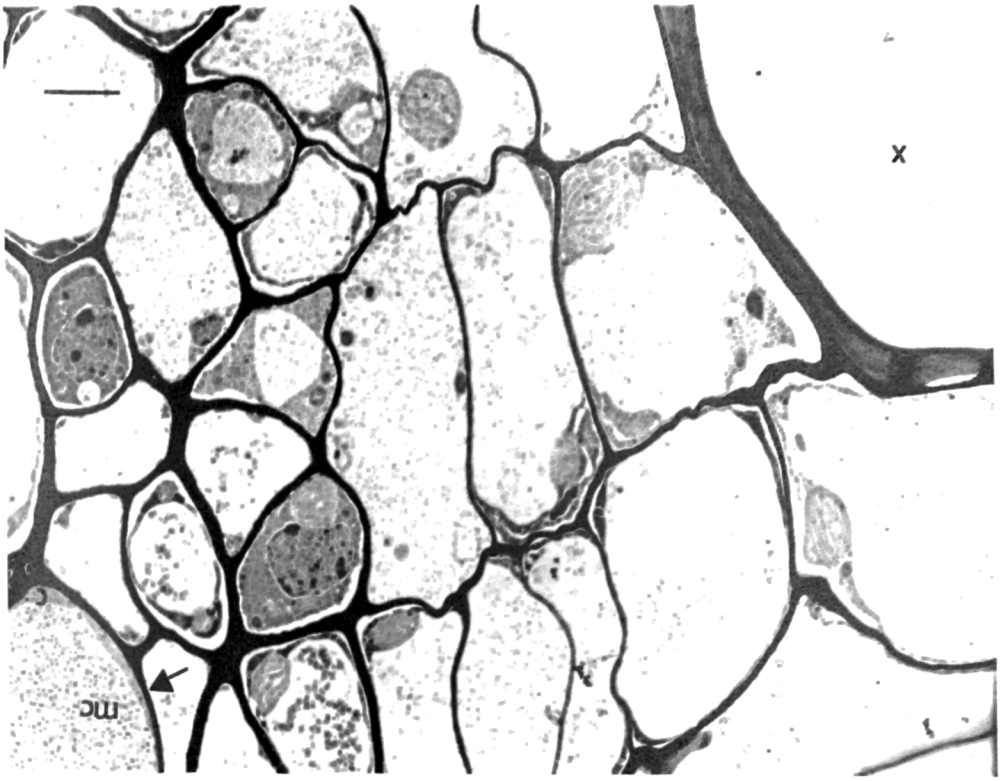
3.2.2.4. Immunogold localization of myrosinase in control and inoculated stems of oilseed rape cv. Cobra with either the virulent isolate 161 of the pathogenic diploid strain viz. *V.d.longisporum* or an avirulent isolate 130 of an haploid strain (tomato) of *V.dahliae.*

Two week-old seedlings were inoculated by the root-dipping method with either the virulent isolate 161 of the pathogenic diploid strain viz. *V.d.longisporum* or an avirulent isolate 130 of a non-pathogenic haploid strain (tomato) of *V.dahliae* as described in section 2.5.2.2.1. Stem sections for all treatments were prepared for EM studies at 18 days post-inoculation; (both conventional and that involving immunogold labelling) as described in section 2.5.2.2.2.1 (fixation b). Semi-thin sections were cut, collected on glass slides, and viewed after staining with 1% Toluidine blue in Borax. The inoculated tissue was examined for hyphal cells which stained dense blue and when hyphae were localized in the inoculated tissue the resin block was trimmed down for ultra-thin sections (100nm). Ultra-thin sections on grids were prepared for EM studies as described in section 2.5.2.2.4. (with procedure 2). The KO89 antibody was used in 1/15.000 dilution.

In control stems, myrosinase was localized in myrosin cells located in the phloem. One-three cells were found to contain the enzyme. Subcellularly, the enzyme was localized inside the vacuole, and either occupied the whole vacuole, or was associated with a sparse amount of proteinaceous material. No myrosin cells or any type of labelling were found in the xylem of control sections. The enzyme was restricted to vacuoles of specific cells in the phloem.

In '161-inoculated seedlings' myrosinase was also localized in myrosin cells in phloem cells. One-three cells were found to contain the enzyme which was again found inside the vacuoles. Fungal spores were found in the xylem vessels and the contact xylem parenchyma cells responded to the local presence of the pathogen showing a positive reaction to the labelled antibody. Most of the xylem parenchyma cells had been damaged by the fungus whereas myrosin cells were intact as compared with the other phloem and xylem parenchyma cells.



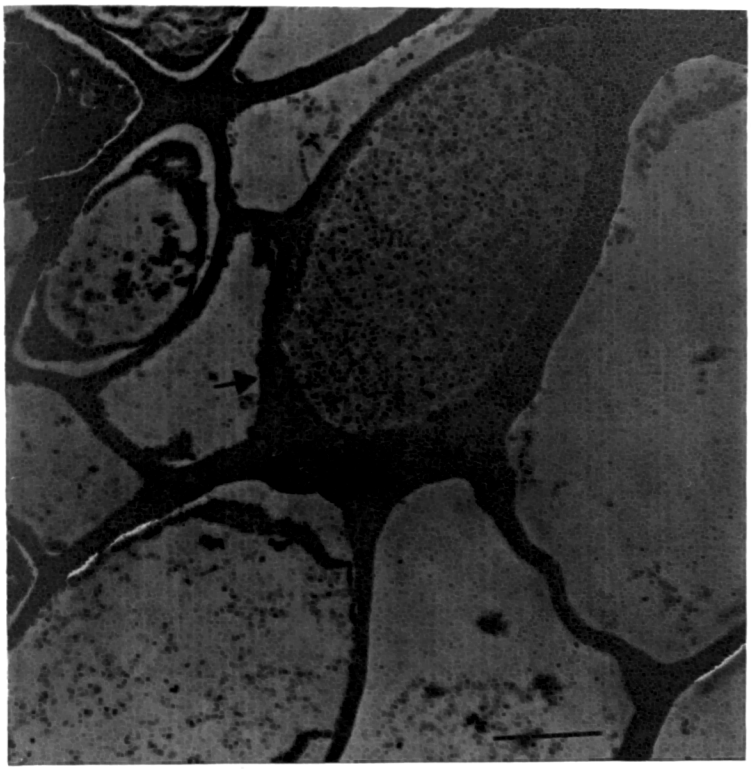
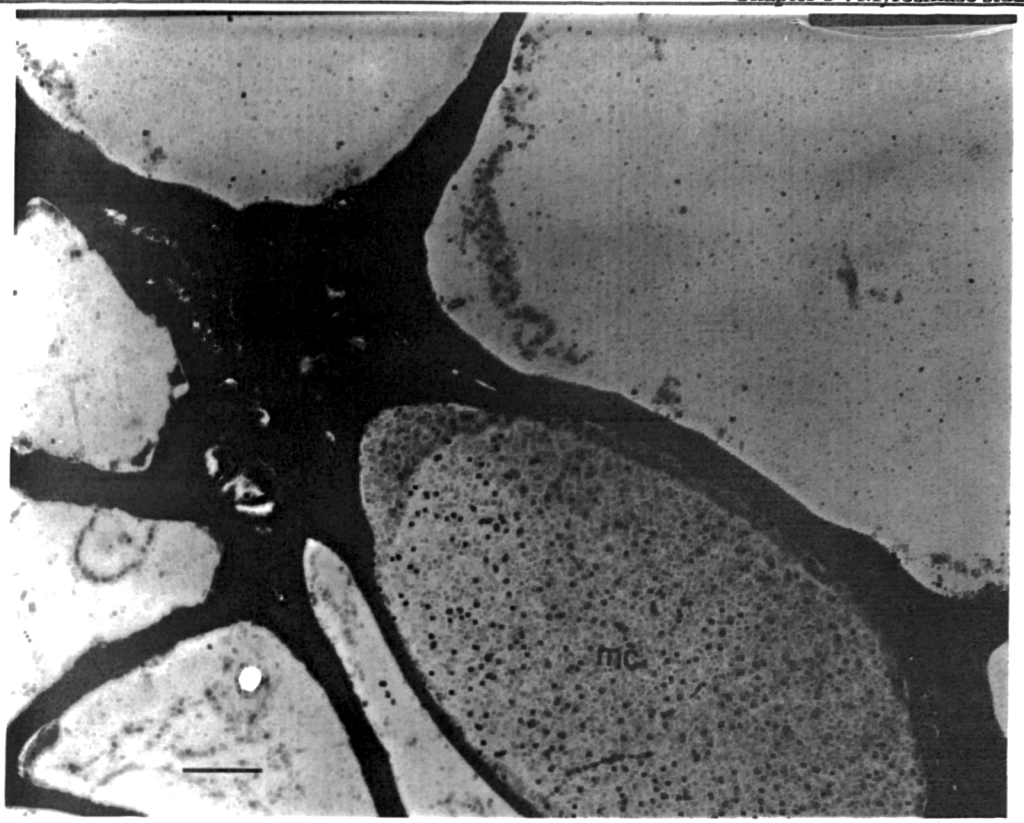


Plate-3.81- Stem section of a control stem, 18 days post-inoculation, labelled with anti-myrosinase (K089) and colloidal gold (30nm); post-stained with 1% aqueous osmium tetroxide, followed by 2% aqueous uranyl acetate (10min) and Reynolds' lead citrate for 3 min. One myrosin cell can be seen in the phloem with dense labelling inside the vacuole which occupies the entire cell. No labelling is seen in the cytoplasm of this cell. Xylem parenchyma cells lack labelling. Magnification X5K. Bar: 2µm. Section has been viewed with a Jeol 1200 EX (Japan) electron microscope at 60V.

Plate-3.82- Detail of the myrosin cell above (arrow). Myrosinase is clearly restricted to the vacuole of this cell. Cytoplasm (c) has no label. Magnification X8K, bar: 2µm.



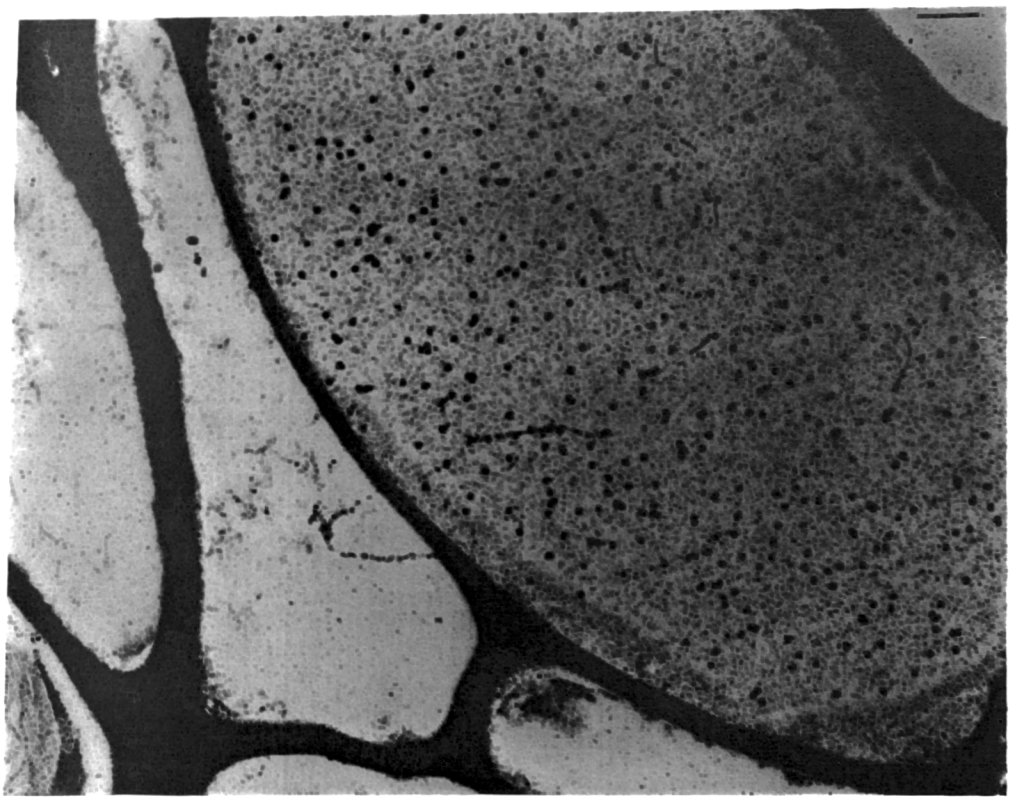


Plate-3.83- Higher (12K) magnification of the phloem myrosin cell above, revealing the specific labelling within the vacuole. Bar: 1µm

Plate-3.84- Detail of the vacuole above in a phloem myrosin cell. The enzyme is uniformly distributed inside the vacuole. Magnification X20K, bar: 500nm.

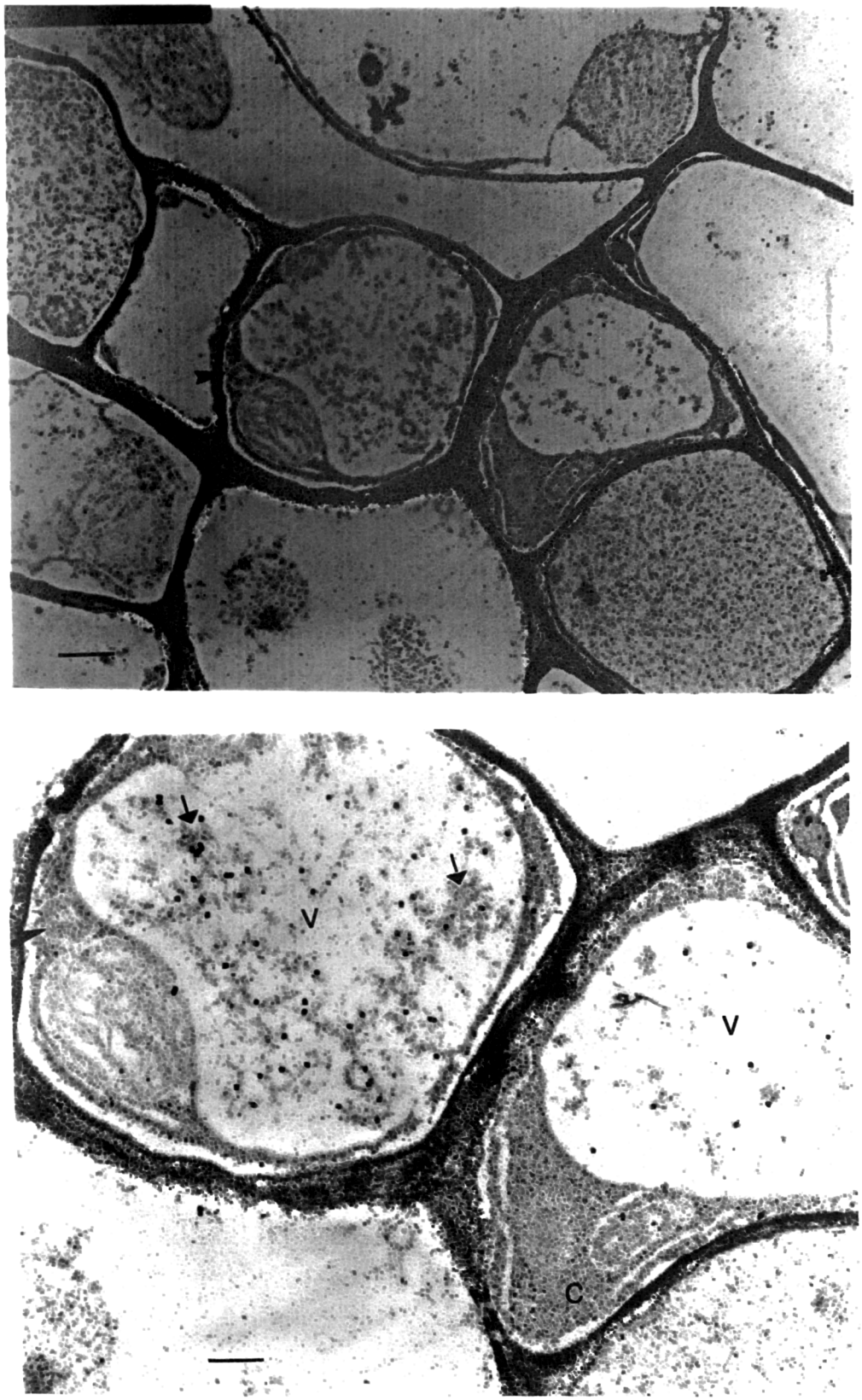
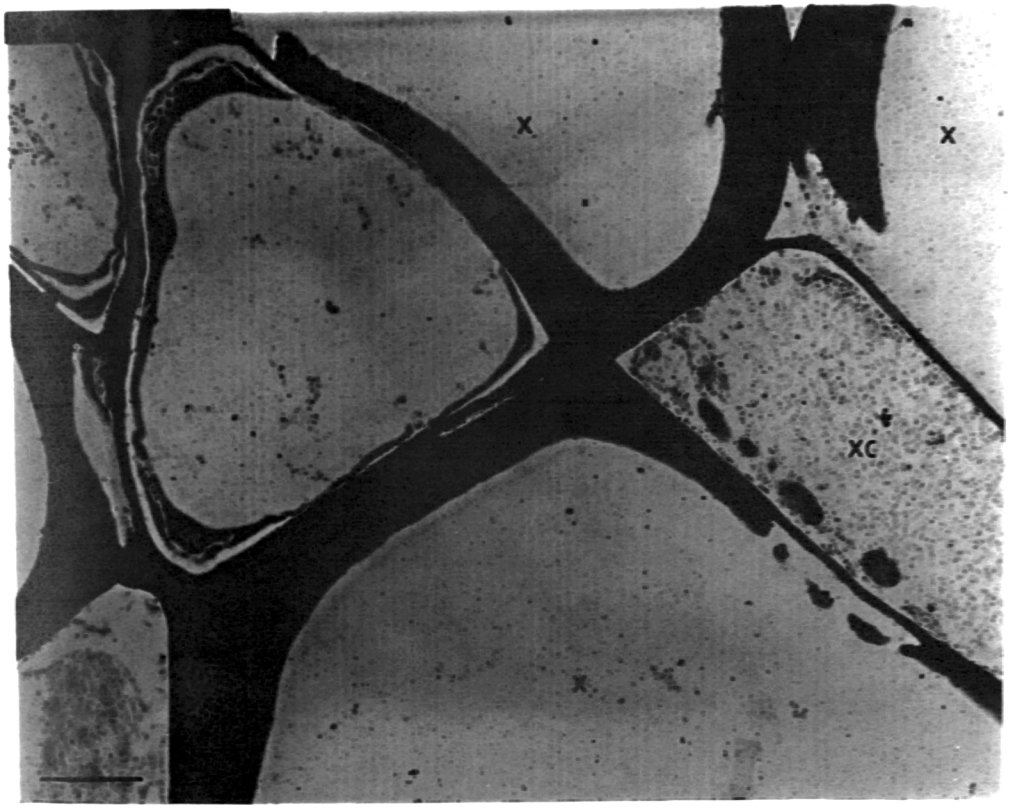


Plate-3.85- Phloem cells show some labelling in the vacuole associated with sparse proteinaceous material inside the vacuole. Magnification x10K, bar: 1µm.

Plate-3.86- Detail of the phloem cell above with specific labelling. Gold particles are associated with this sparse proteinaceous material (arrow) inside the vacuole. Magnification x20K, bar: 500nm.



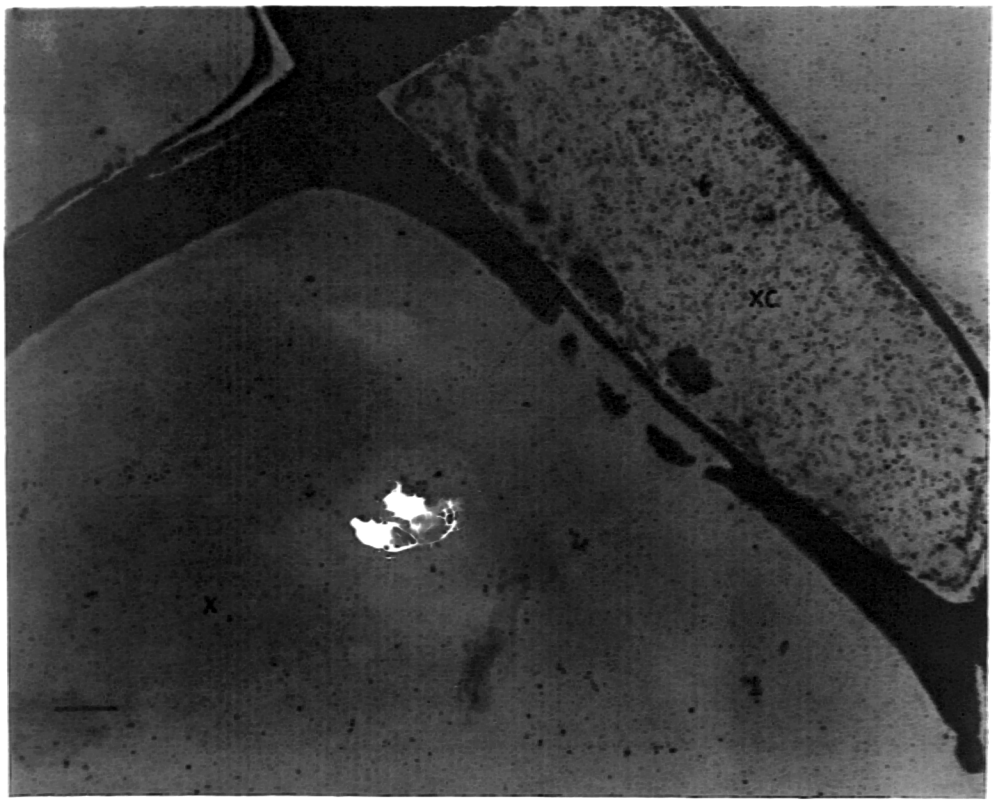


Plate-3.87- Empty xylem vessels. No labelling is seen in a contact xylem parenchyma cell (xc). Magnification x8K, bar: 2μm.

Plate-3.88- Detail of the xylem contact cell above. No labelling can be detected in this cell of the stem of a control oilseed rape seedling. Magnification X10K, bar:1μm.

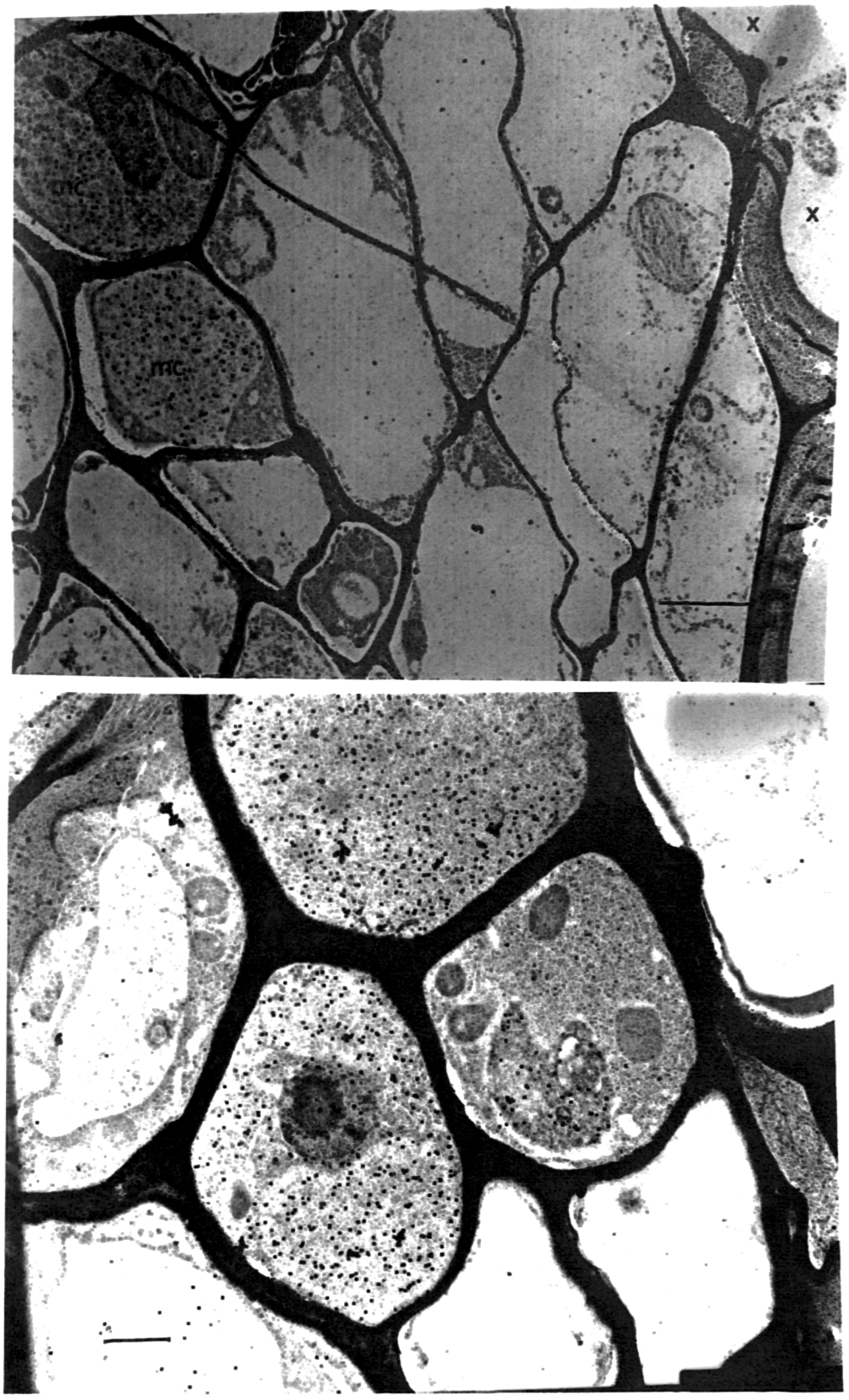
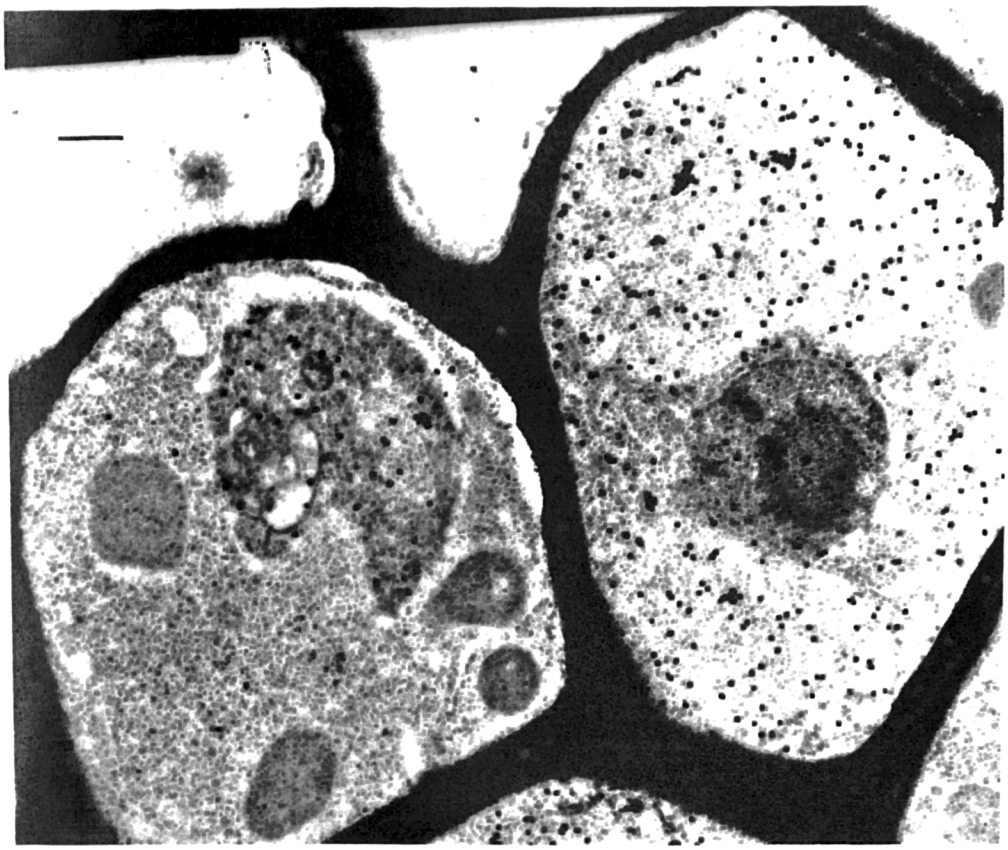


Plate-3.89- Two myrosin cells in the phloem of the stem of control oilseed rape seedling. No myrosinase can be detected in the xylem parenchyma cells. Magnification x8K, bar: 2µm.

Plate-3.90- Three myrosin cells in phloem in stem of 161-inoculated seedling. Magnification X12K, bar: 2µm.



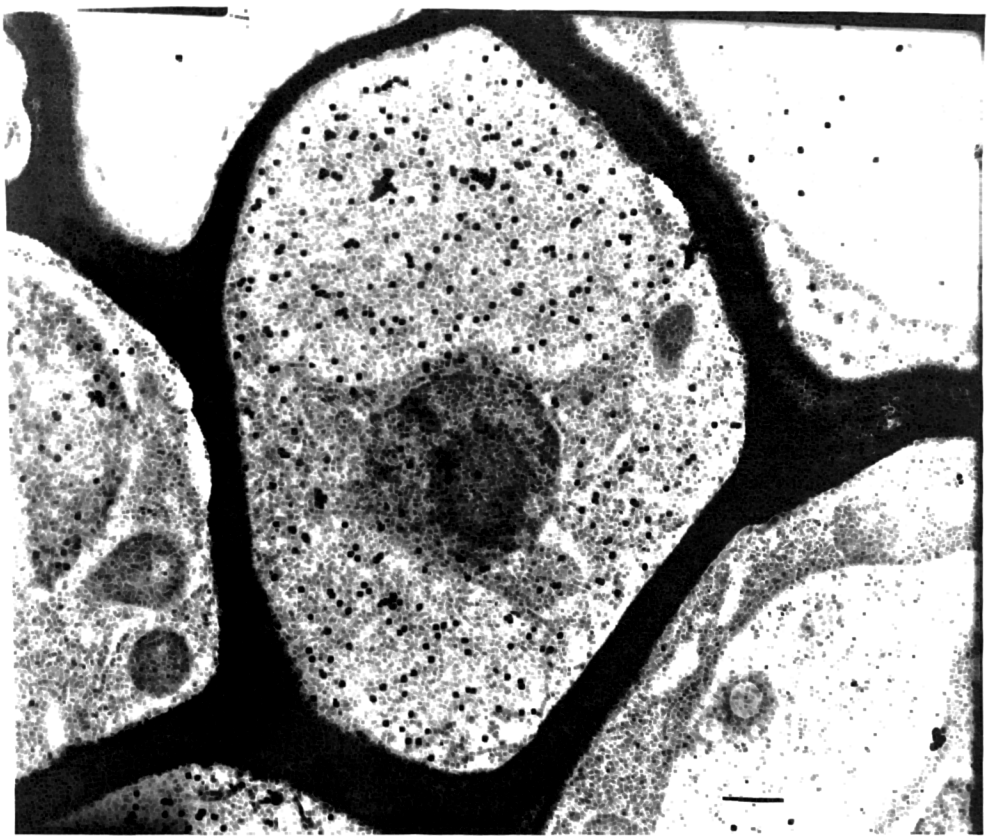
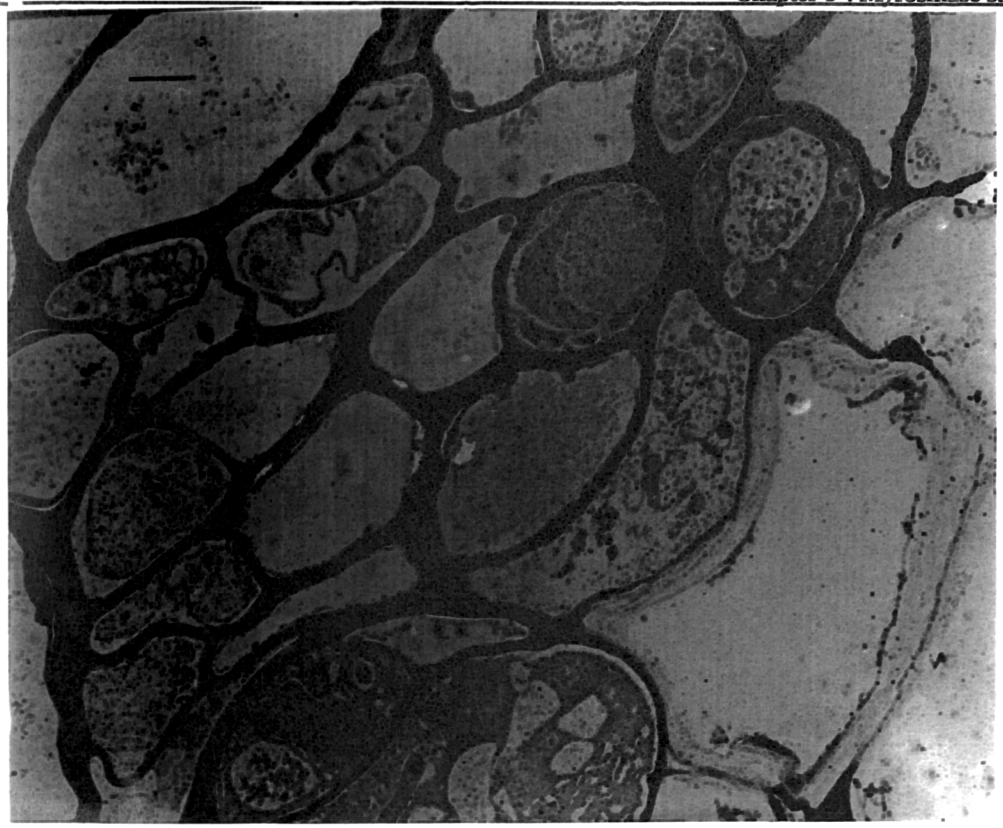


Plate-3.91- Detail of two of the phloem myrosin cells above. The enzyme is restricted to the vacuole. Magnification X12K, bar: 1µm.

Plate-3.92- Higher magnification of the myrosin cell above in Fig-3.91-. Magnification x20K, bar: 500nm.



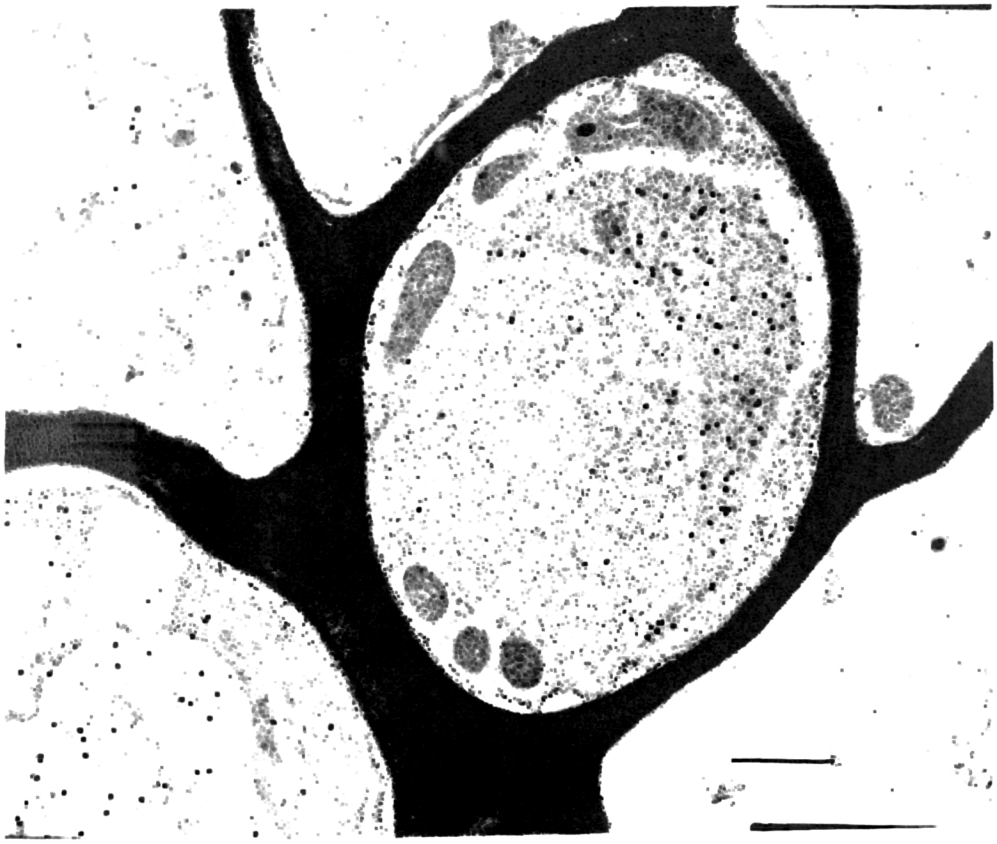
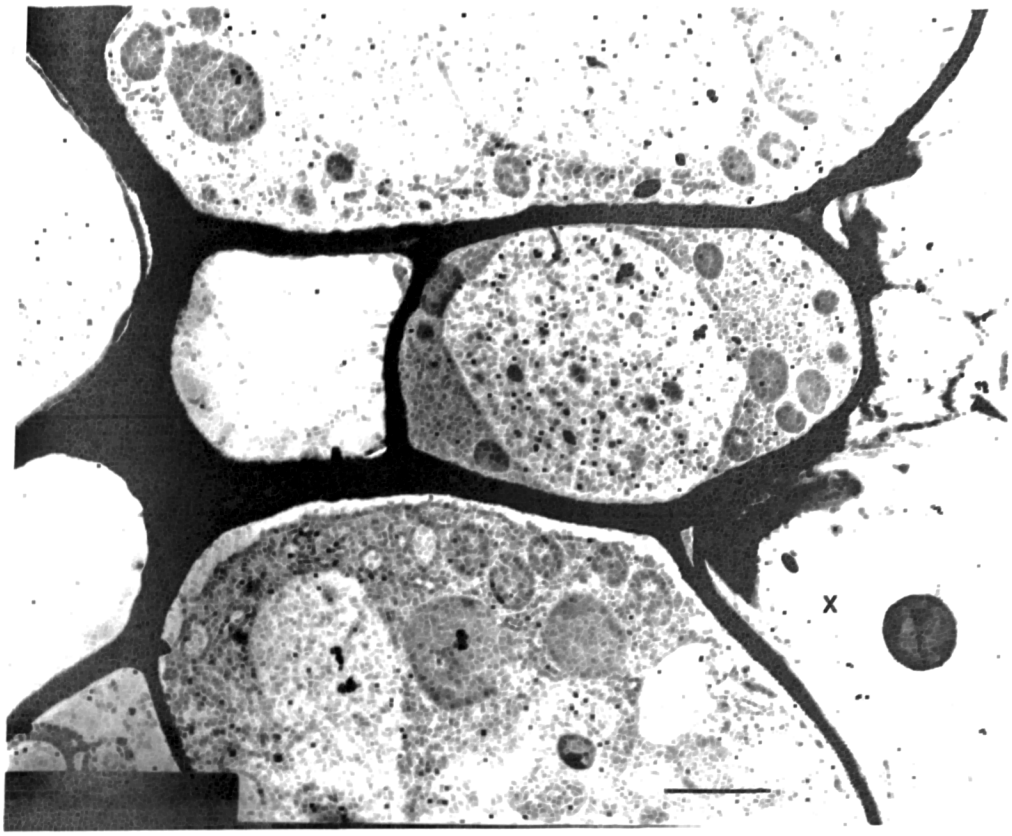
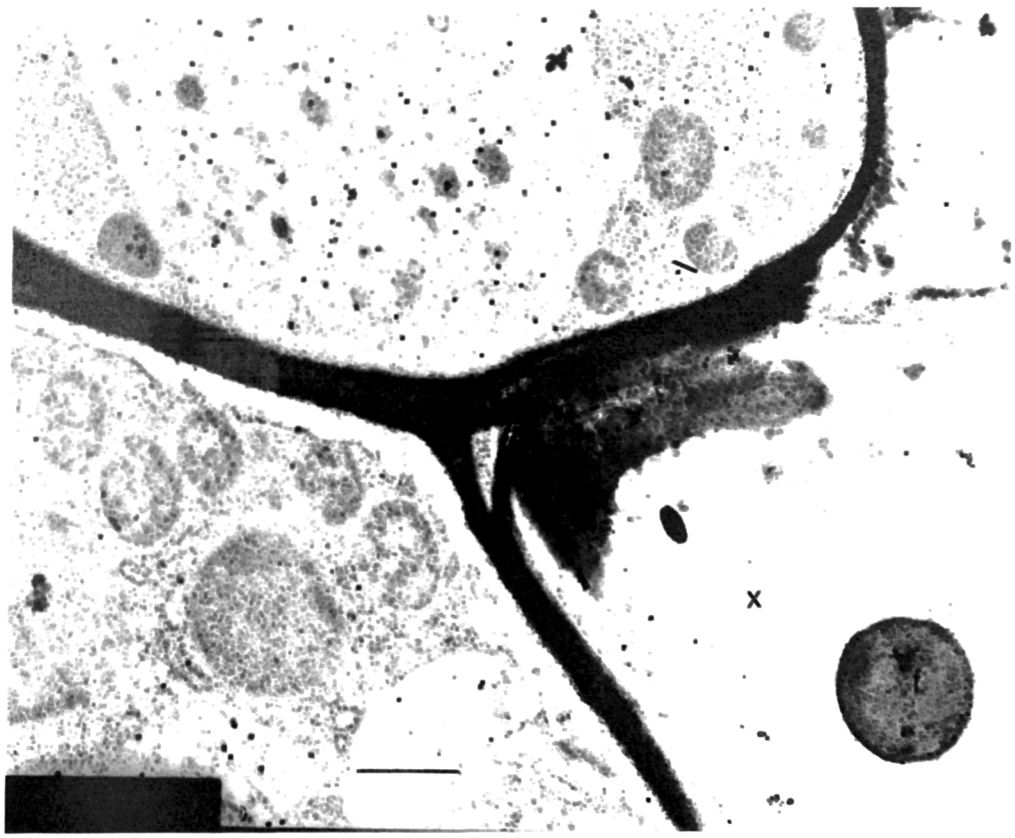


Plate-3.93- Micrograph of a section of the stem of a '161-inoculated' plant. Some xylem parenchyma cells have been disrupted, and some show a reaction to the presence of the fungus. One myrosin cell can be seen in the phloem tissue. Magnification X5K, bar: 2μm

Plate-3.94- Detail of the phloem myrosin cell above. The enzyme is restricted to the vacuole. Magnification x16K, bar: 1μm.





ıte-3.95- Fungal spore in xylem vessel (x) of '161 inoculated' seedling. Xylem parenchyma contact cell onds to the presence of the fungus, gold label can be seen inside its vacuole. Magnification X8K, bar:1µm.

te-3.96- Detail of the fungal spore and myrosin cell above. Magnification X 16K, bar: 1µm.

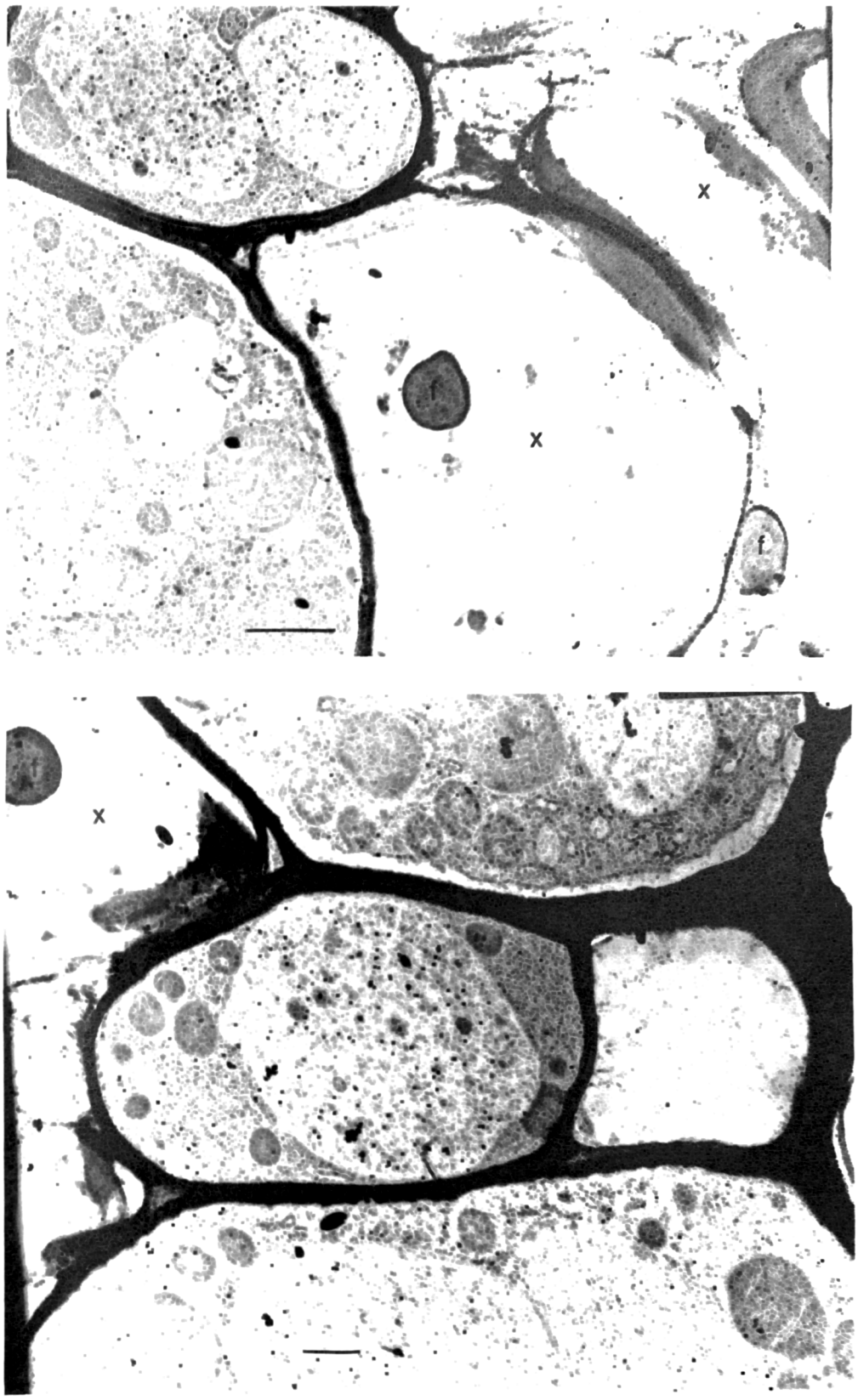


Plate-3.97- Fungal spores of isolate 161 of the diploid strain *V.d.longisporum* in the xylem vessels (x) of oilseed rape seedling. Xylem contact parenchyma cell responds to the presence of the fungus and shows positive labelling in the vacuole.. Magnification X 8K, bar: 2µm.

Plate-3.98- Detail of a xylem parenchyma contact cell of 161-inoculated oilseed rape seedling with immunogold labelling of myrosinase inside the vacuole. Magnification X 10K, bar: 1µm.

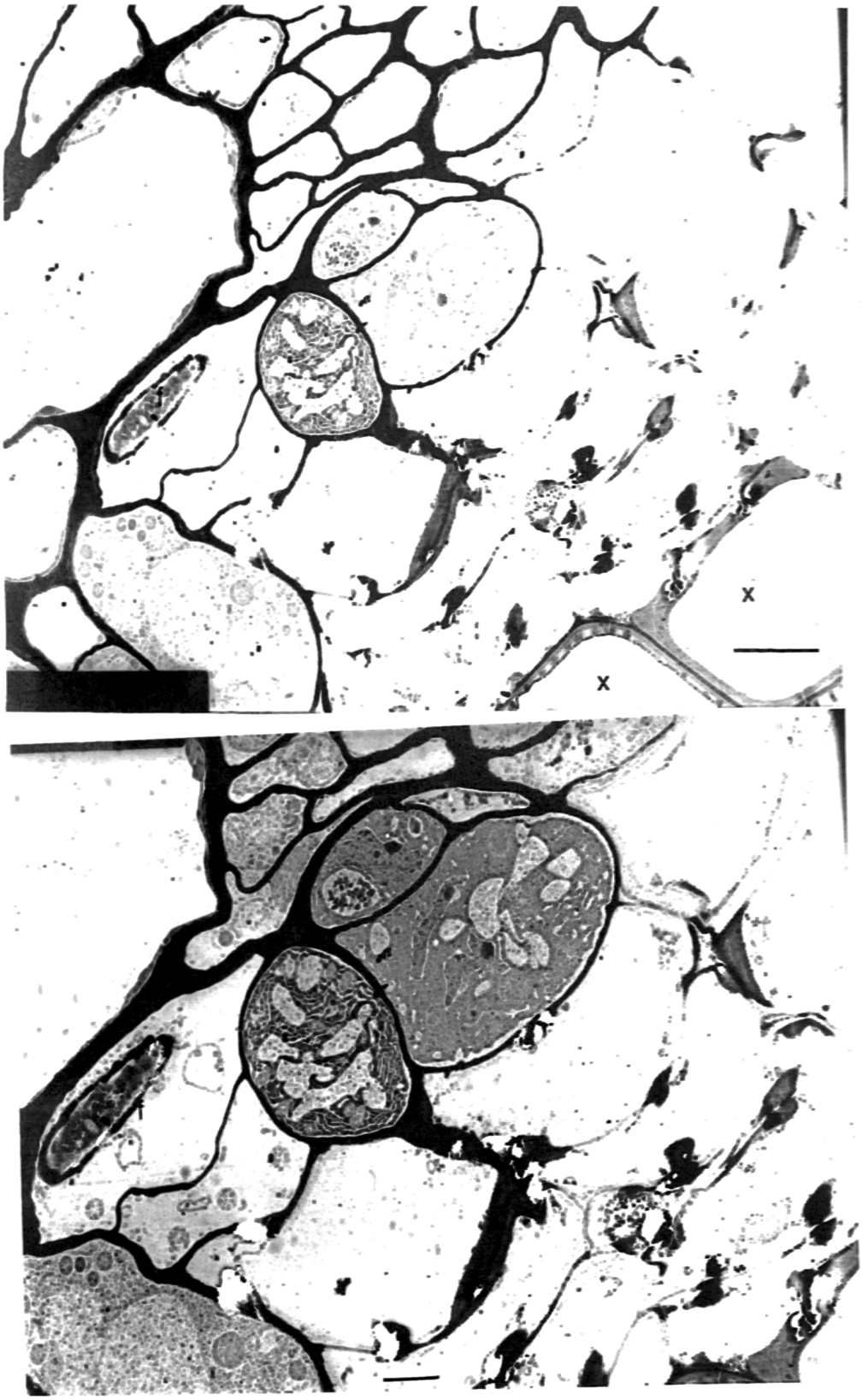
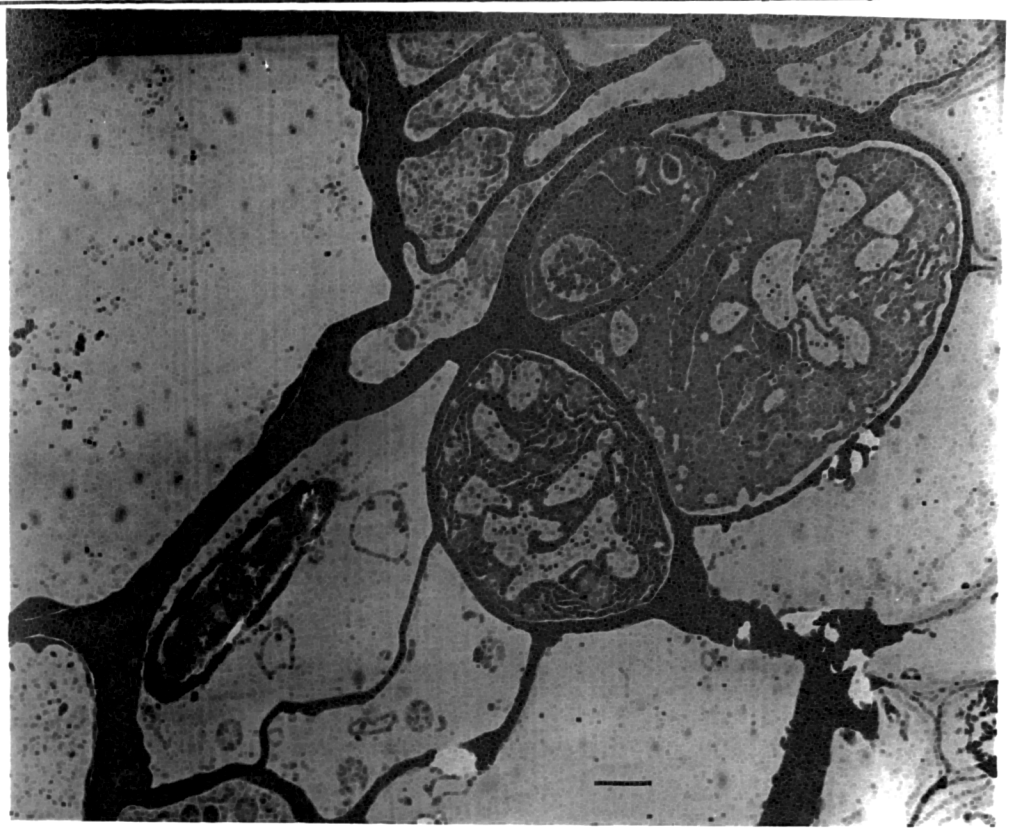


Plate-3.99- Fungal spore of isolate 161 of the diploid strain 161 in xylem parenchyma cell. A number of cells of the xylem parenchyma and xylem vessels are completely disrupted. Cells are extensively deformed and show their damaged cell walls. Magnification X3K, bar: 5µm.

Plate-3.100- Detail of the fungal spore and damaged xylem parenchyma cells above. Magnification X 5K, bar: 2µm



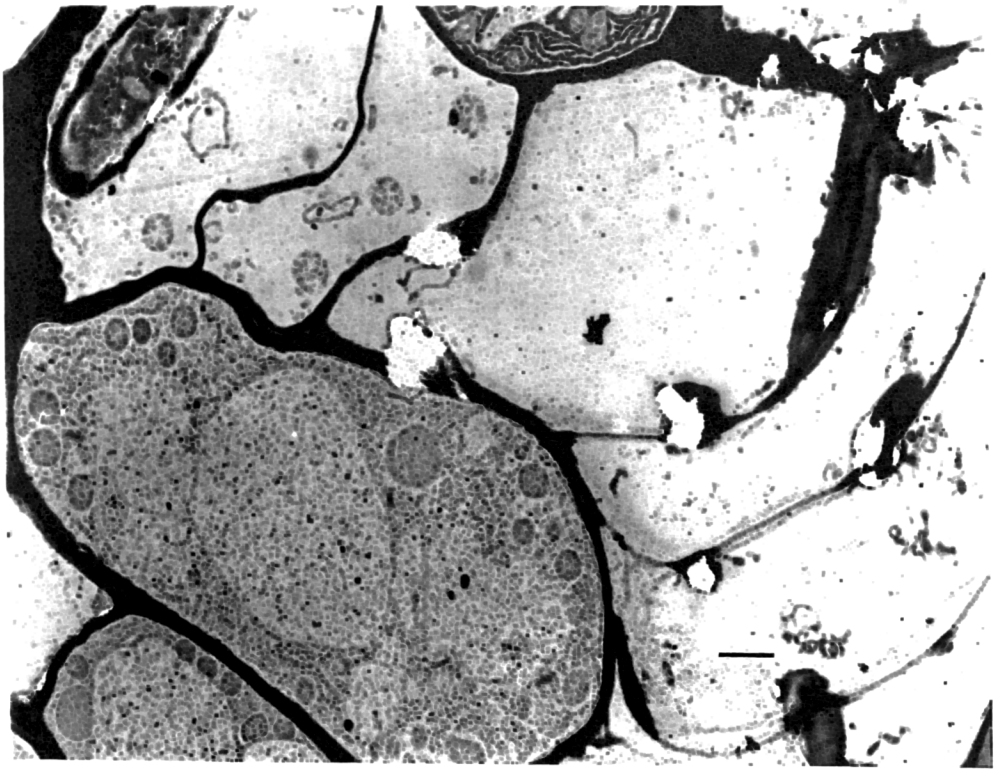


Plate-3.101- Contact xylem parenchyma cell of stem of '161-infected' oilseed rape seedling shows some labelling inside the vacuole and a very large number of dilated cisternae of ER as observed in myrosin cells in healthy young radicles. Magnification X5K, bar: 5K.

Plate-3.102- Detail of the xylem parenchyma cells above which are severely damaged. An increased number of mitochondria can be seen in one xylem parenchyma cell, indicating that this cell is metabolically active suggesting an active HR reaction. Magnification X10K, bar: 2μm.

3.2.2.5. Conventional staining of petioles and stems and immunogold labelling of stems of control and '161 inoculated' plants of oilseed rape cv. Cobra 20 days post-inoculation.

Seedlings of oilseed rape cultivar Cobra were inoculated at 2 weeks-old by root dipping (in $1X10^6$ spores/ml) and petiole and stem segments were fixed at 20 days post-inoculation in 2.5% glutaraldehyde in 0.05M cacodylate buffer overnight, and followed by post-fixation with 1% OSO_4 in the same buffer for conventional EM studies.

The xylem vessels, xylem parenchyma cells, and related intercellular spaces of the xylem of stems and leaf petioles were full of fungal spores and hyphae. Extensive degeneration of cell walls, total disruption of membranes, cytoplasm and organelles of xylem parenchyma cells was observed in 161-infected petioles and stems. Hyphae grew from one vessel to an adjacent vessel through cells walls and through degraded cell wall material. Hyphae as they entered xylem cell walls were restricted at the site of penetration, but resumed a normal dimension after penetration.

Extensive cell wall degradation was observed, indicating release of c.w.d.e.s. by the fungus. An electron-dense dark coating material on vessel walls of xylem and pit fields was observed. Also, fungal spores and hyphae in stems were coated by a fibrillar electron-dense material which seemed to connect the fungus with the host cells.

Xylem vessels and xylem parenchyma cells had been seriously deformed; instead of the normal, almost spherical shape they showed an abnormal, elongate shape. Vessel occlusions were observed in vessels which lacked the fungus but were close to adjacent fungal infected vessels.

Additionaly eedlings of oilseed rape cultivar Cobra were inoculated at 2 weeks-old by root dipping (in $1X10^6$ spores/ml) and 20 days post-inoculation stems prepared for electron microscopic studies (immunocytological) as described in section 2.5.2.2.2. (fixation-a-, and immunogold labelling procedure-1-). In control stems, myrosinase was localized in myrosin cells located in the phloem. Subcellularly , the enzyme was localized inside the vacuole, no label was found in contact xylem parenchyma cells (plate 3.1210). In 161-infected seedlings myrosinase was also localized in phloem cells, in their vacuoles. Fungal spores were found in xylem vessels and the contact xylem parenchyma cells responded to the local presence of the fungus showing a positive reaction to the labelled antibody (M1), (plates 3.121, 3.122). Contact xylem parencyma cells that were adjucent to vessels that lecked any fungul spores did not show any positive reaction to the labelled antibody (3.123).

Plate-3.103- Spores and hyphae in a transverse section of a petiole of '161- inoculated' oilseed rape plant cv. Cobra, 20 days post-inoculation. Seriously deformed cell and damaged cell walls can be seen. Magnification X7K.

Plate-3.104- Hypha in a completely disrupted xylem vessel (x) penetrating a degraded wall. The hypha narrows (arrow) at the site of penetration. Extensive degradation of pit fields in the presence of invading hyphae can be seen (head-arrow). Magnification X10K.

Plate-3.105- Constriction of hyphae at the point of penetration which attains its normal dimension after penetration (arrow). Magnification X14K.

Plate-3.106- Detail of the penetration site above in Plate-3.106-. Magnification X40K.

Plate-3.107- Detail of penetration site in Plate-3.104-. Magnification X34K.

Plate-3.108- Attachment of a fungal hypha on cell wall. Magnification x34K.

Plate-3.109- Penetration of a cell wall by a fungal hypha.The hyphae is restricted at the site of penetration (arrow) and increases again in diameter after penetration.

Plate-3.110-. Detail of the hyphae above. The penetration point can be distinguished easily. Magnification x80K.

Plate-3.111- Xylem vessels and xylem parenchyma cells of a control oilseed rape seedling petiole section. Magnification X7K.

Plate-3.112- Detail of secondary cell wall of a xylem vessel a control oilseed rape seedling of petiole. Magnification x 34K.

Plate-3.113- Section of a stem of a '161-inoculated' oilseed rape plant cv. Cobra. Cell walls, cytoplasm and organelles show degeneration. Cells have been seriously deformed and xylem vessels (x) that lack the fungus but are close to fungal spores and hyphae show gel occlusions. Fungal spore and hyphae were coated by a fibrillar material. Magnification x 5K.

Plate-3.114- Same section as above. Occlusion of vessel pit can be seen (arrow) by electron-dense material. Fungal propagules are coated by a fibrillar material. A xylem parenchyma contact cell (XPC) is undergoing a metabolically active phase prior to a HRMagnification X 5K.

Plate-3.115- Seriously deformed xylem parenchyma and vessels of a stem of '161 -infected' oilseed rape seedling. Cell walls have been degraded and cells have lost their shape. Magnification x5K.

Plate-3.116- Fungal spores and hyphae of isolate 161 of the pathogenic diploid strain *V.d.longisporum* in a vessel of stem of oilseed rape cv. Cobra. Coating material is seen on vessel walls (smooth) and membrane pits. (bubbly). Also fungal propagules are coated with this fibrillar material. Magnification x8K.

Plate-3.117- Detail of fungal propagules (plate-116-) above which demonstrates the fibrillar appearance of the coating material (arrow). Magnification x 20K.

Plate-3.118- Bubbly coating material (asterisk) on cell walls and fibrillar on fungus (arrow). Magnification x 20K.

Plate-3.119- Attachment of a fungal hypha (f) on a cell wall (cw). Hyphae and cell wall are coated by this fibrillar material that seemingly attaches them to the vessel wall (delimited by arrows), this coating material is missing. Magnification X60K.

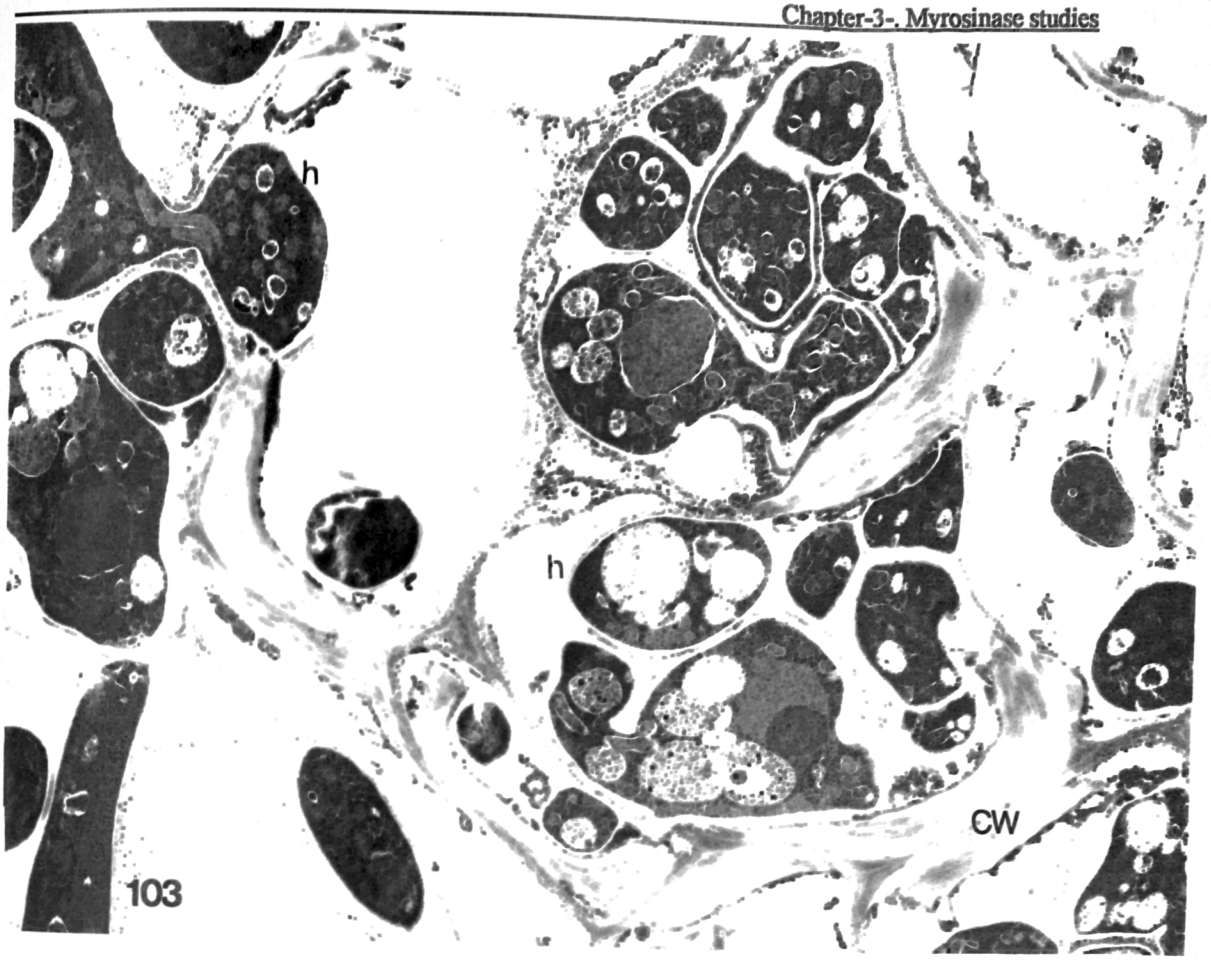
Plate-3.120- Immunogold staining of a section of control stem at 20 days post-inoculation. No colloidal gold is seen in the contact xylem parenchyma cells. The grid supporting the tissue was incubated in primary antibody

M1 with procedure 1 (Results section, page 77) and was observed with a Hitachi H-7000 electron microscope at 75V. (Magnification X18K).

Plate-3.121- Fungal spore in xylem vessel of '161-inoculated' seedling, (20 days after inoculation). Xylem parenchyma contact cell responds to the presence of the fungus, gold labelling can be seen inside its vacuole, especially in specific accumulated bodies (mab). (Magnification X 16K).

Plate-3.122- Detail of the above plate. Coating material is seen on vessel walls and membrane pits (arrow). Gold labelling was mostly found in the vacuole of this reacting xylem contact parenchyma cell. (Magnification X 40 K).

Plate-3.123- Xylem contact parenchyma cell tha was adjacent to a vessel that lacked the fungus did not show positive reaction to the labelled antibody. (Magnification X 16K).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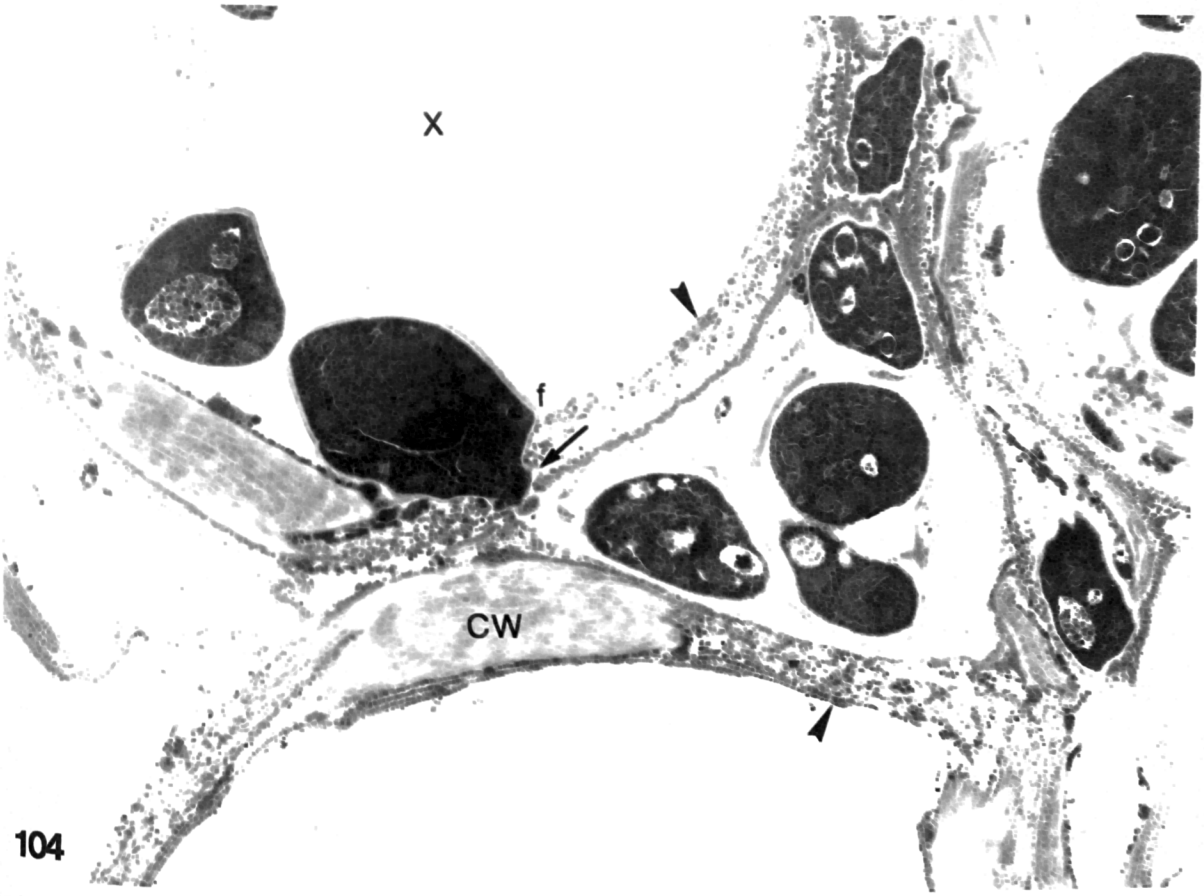
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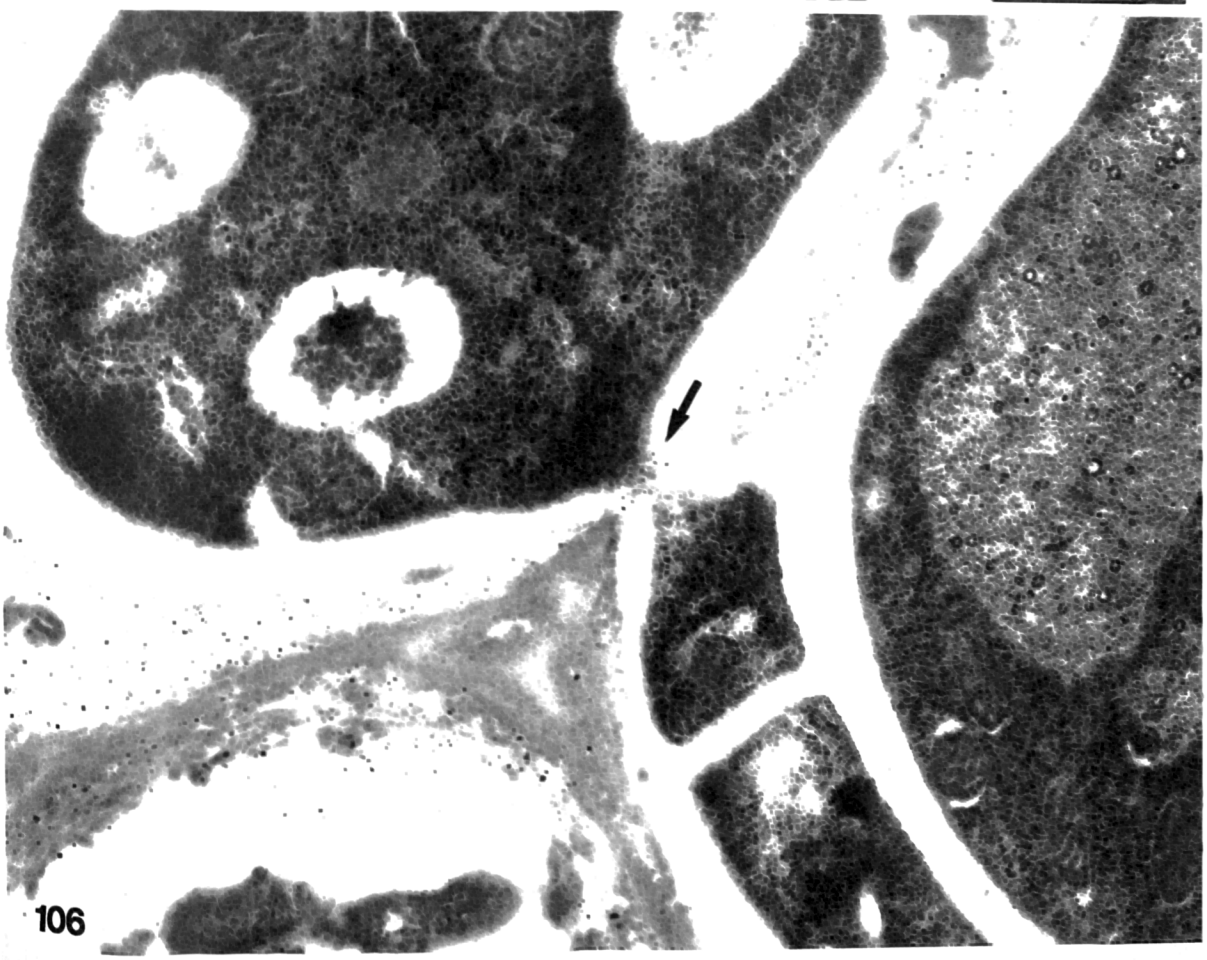

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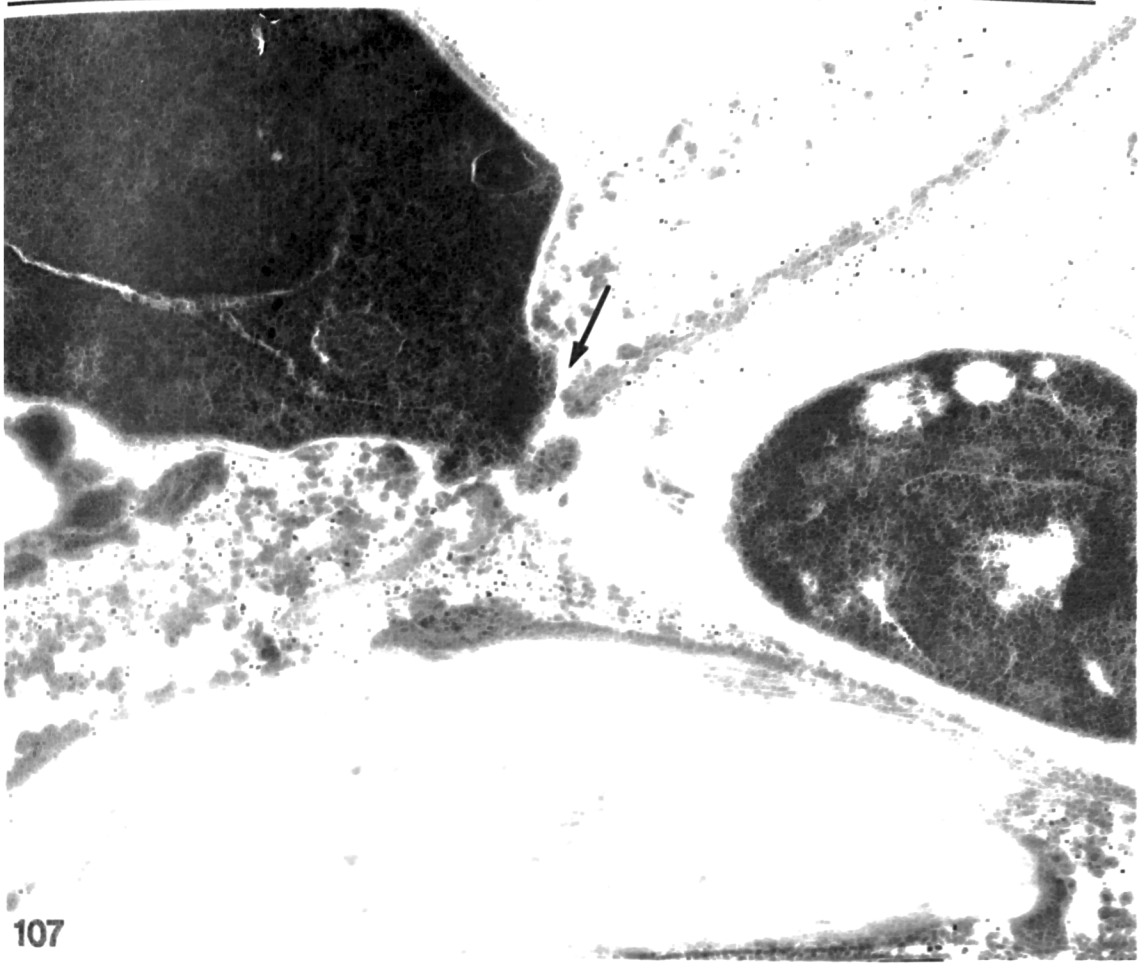

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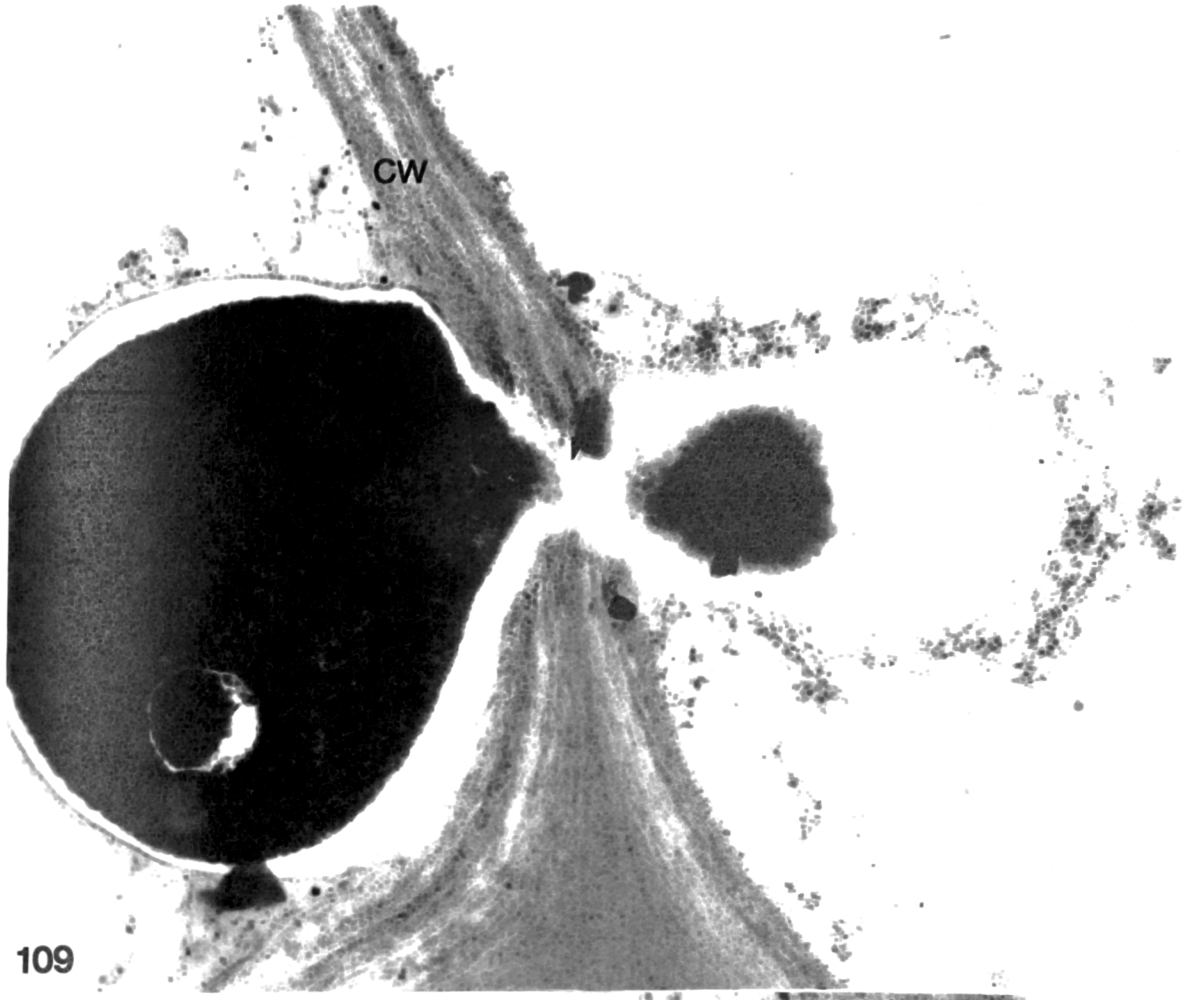

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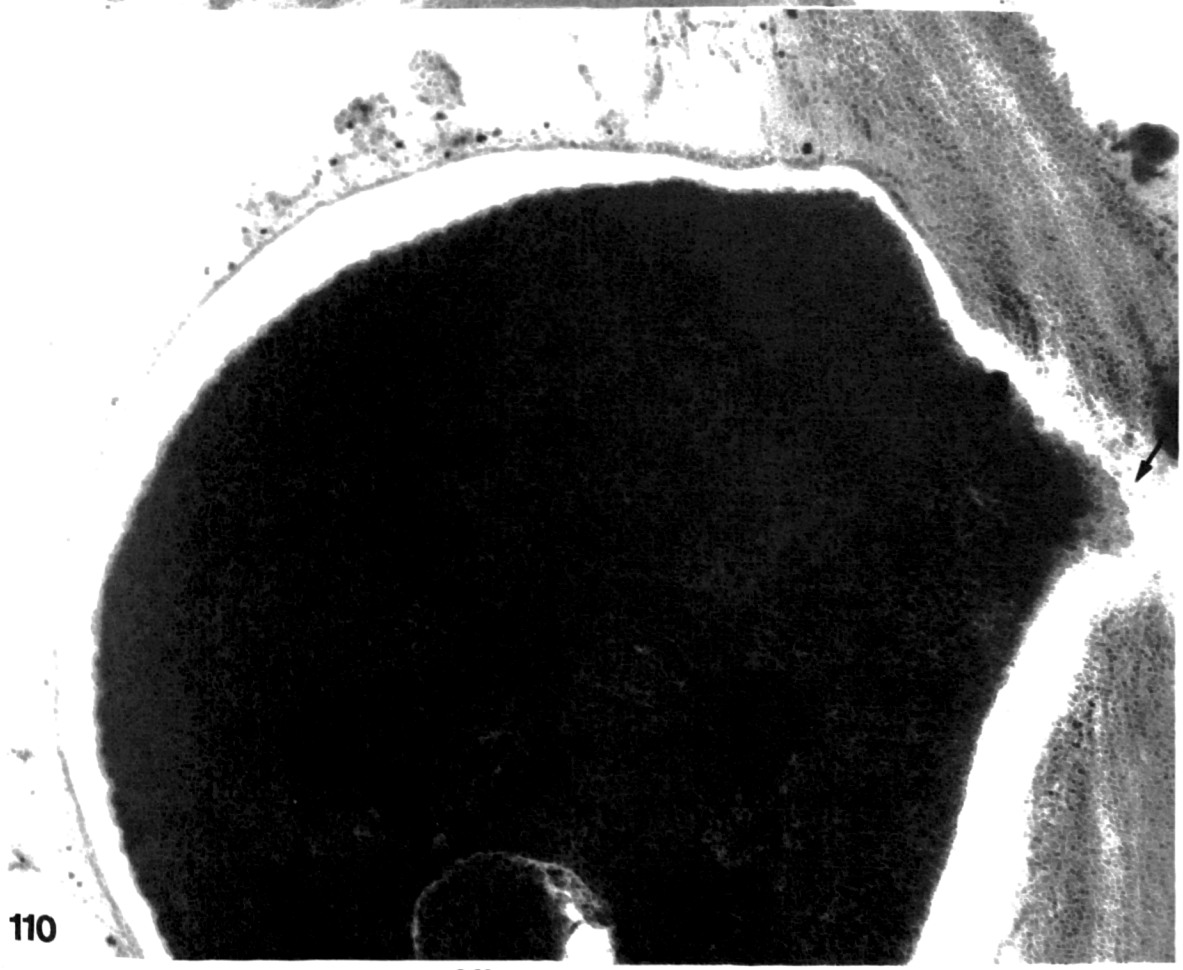

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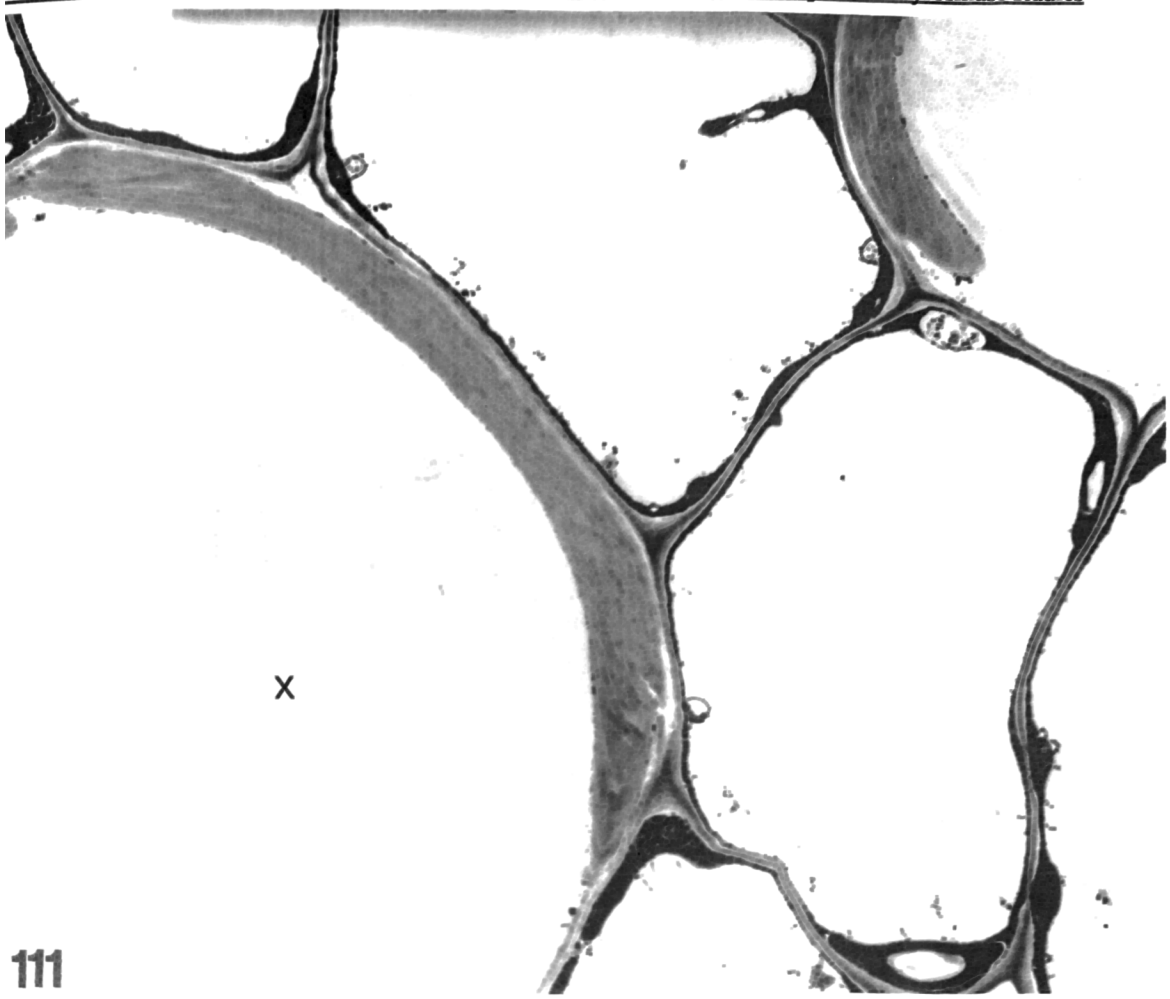

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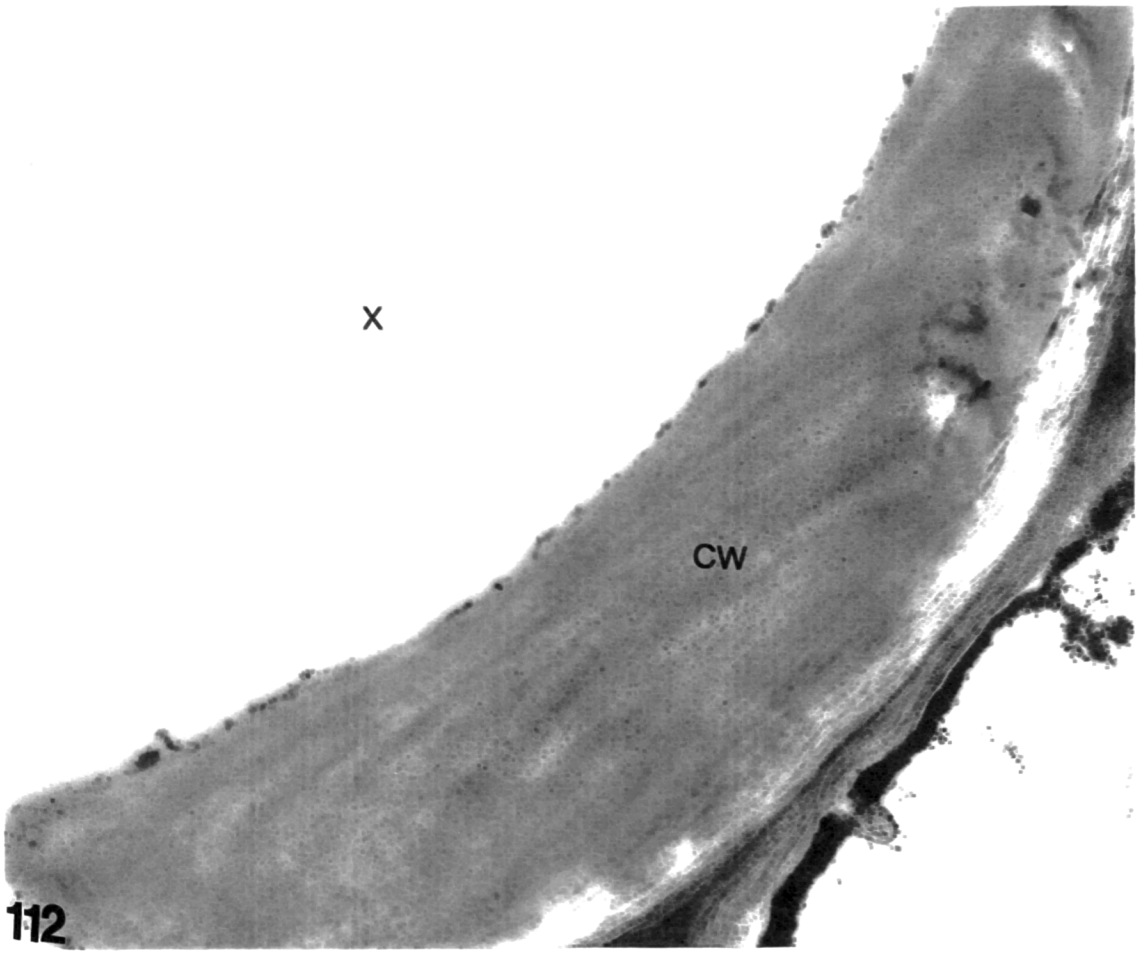

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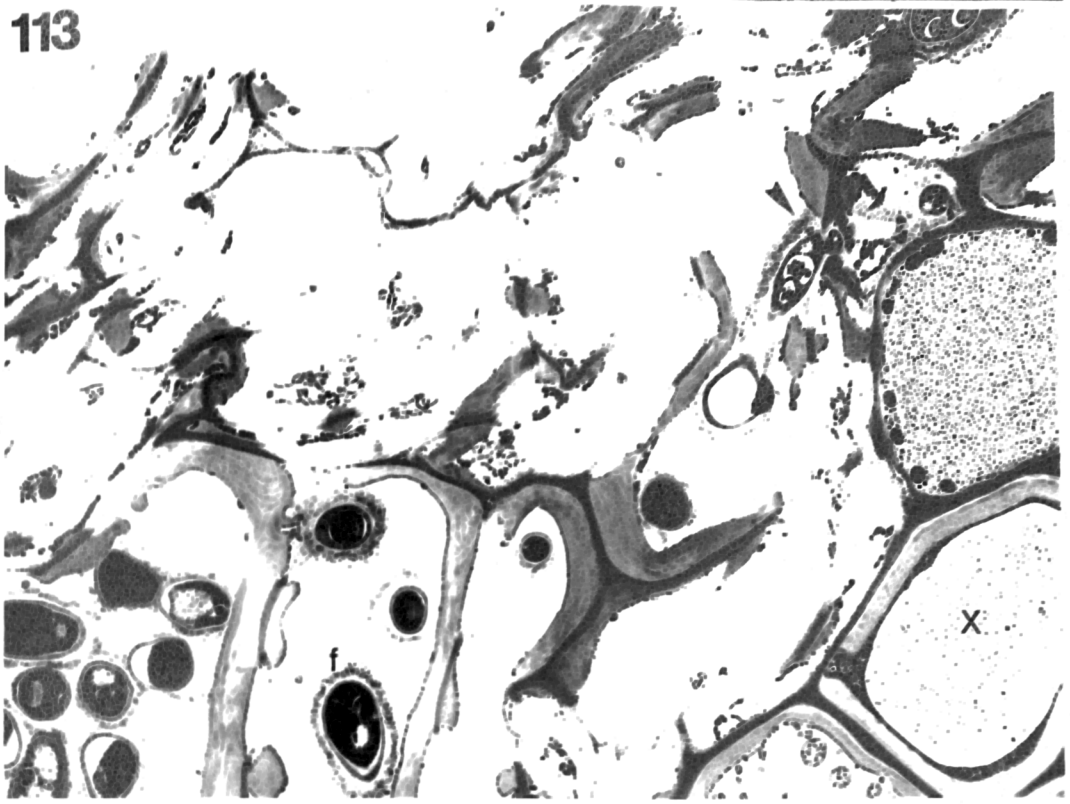
113
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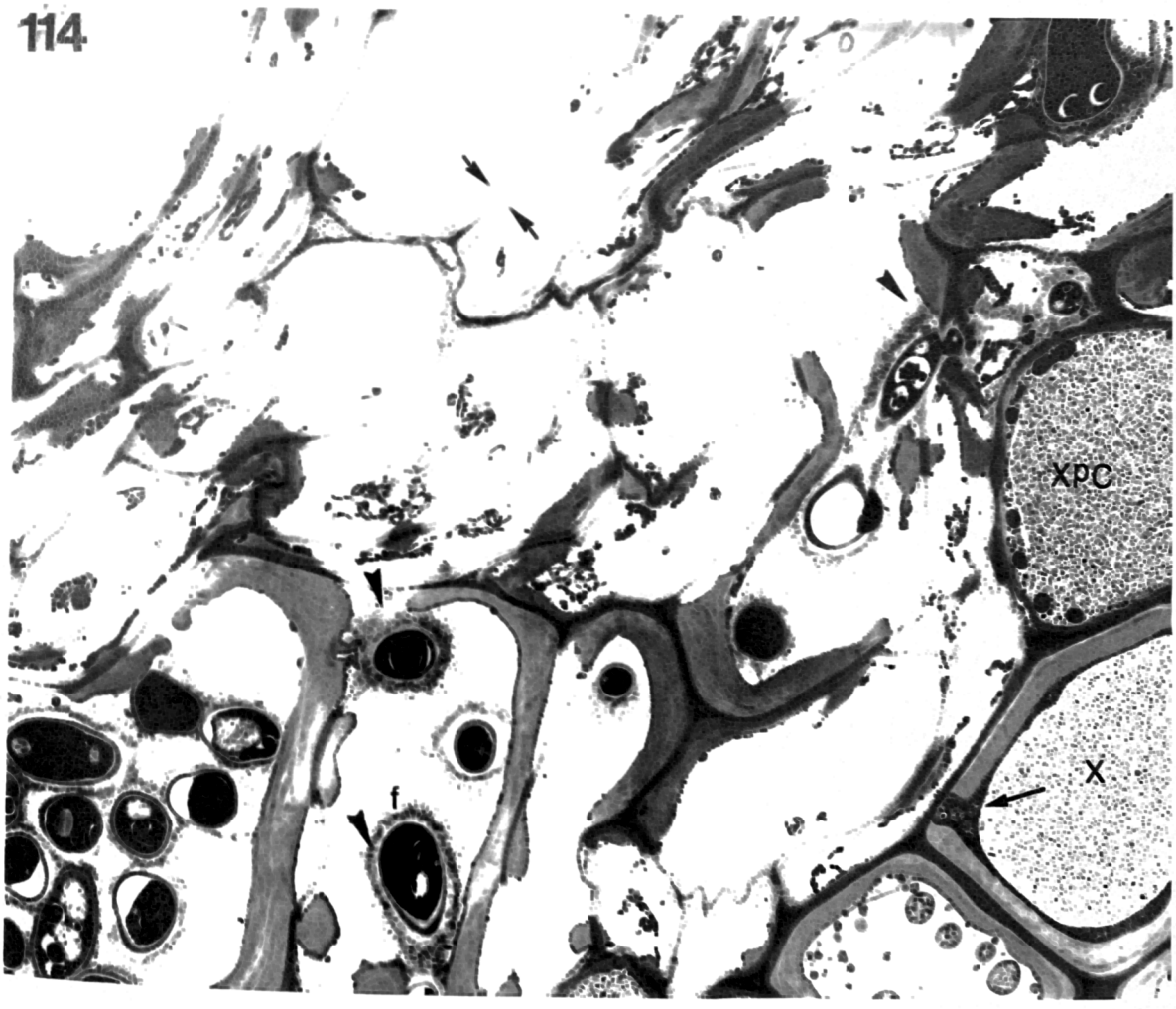
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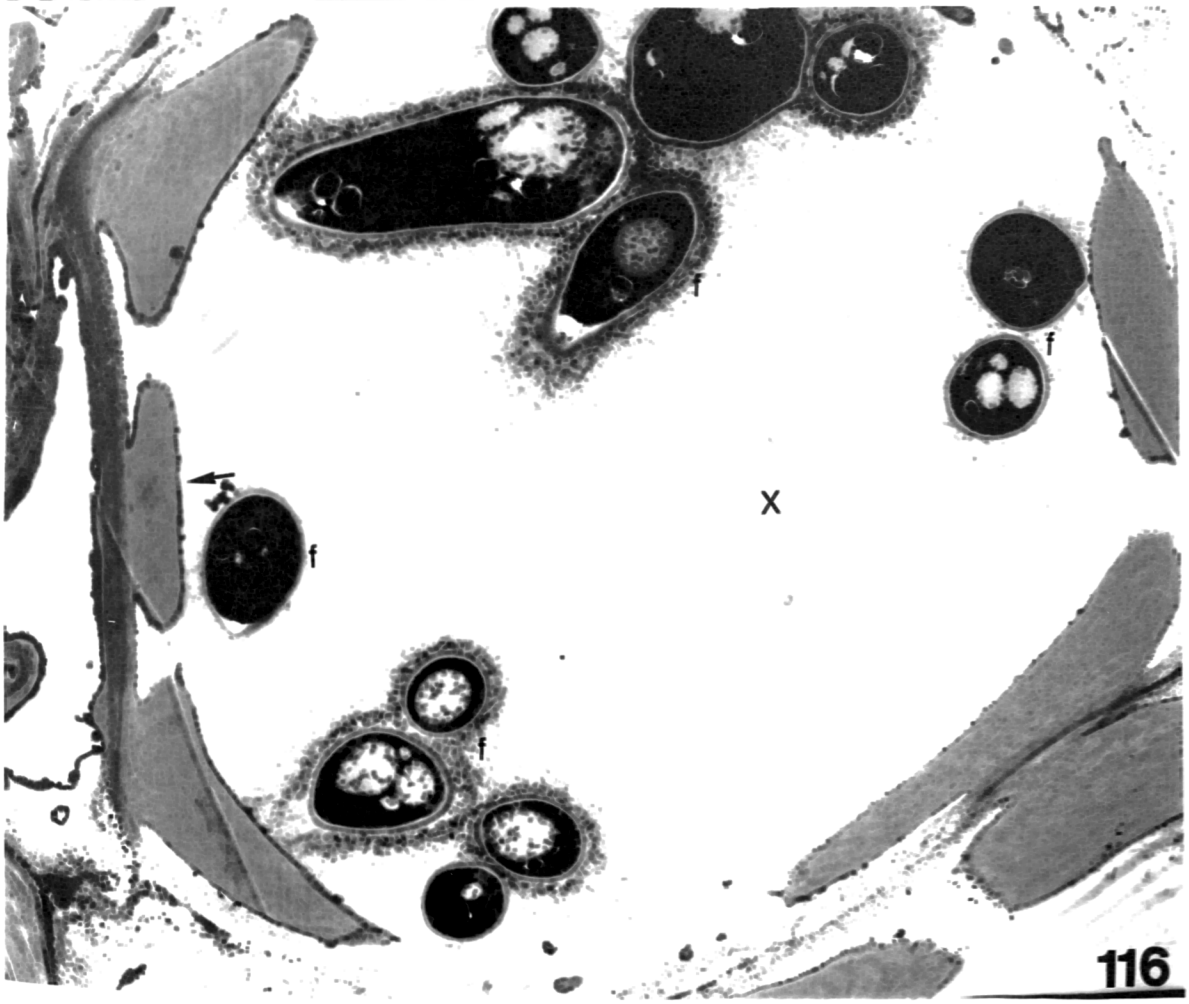

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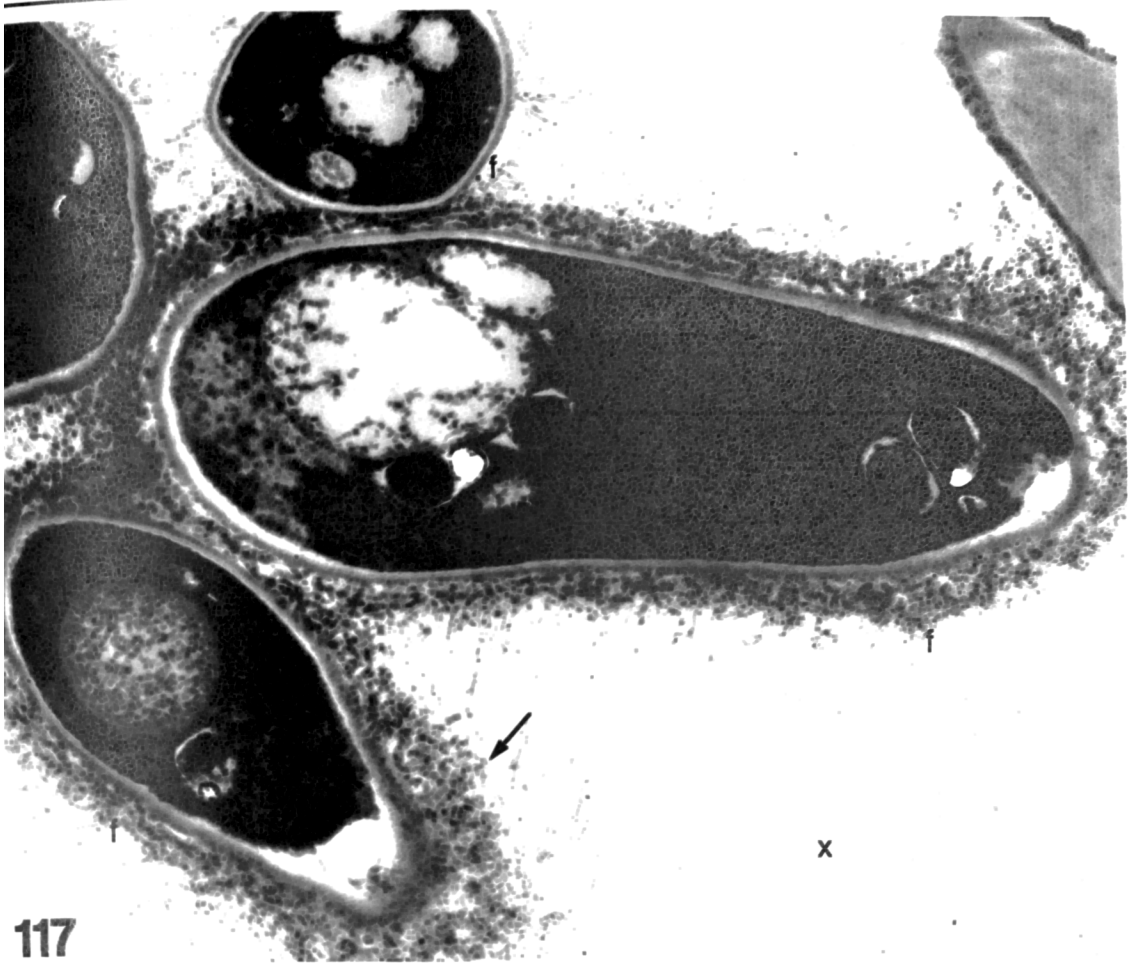

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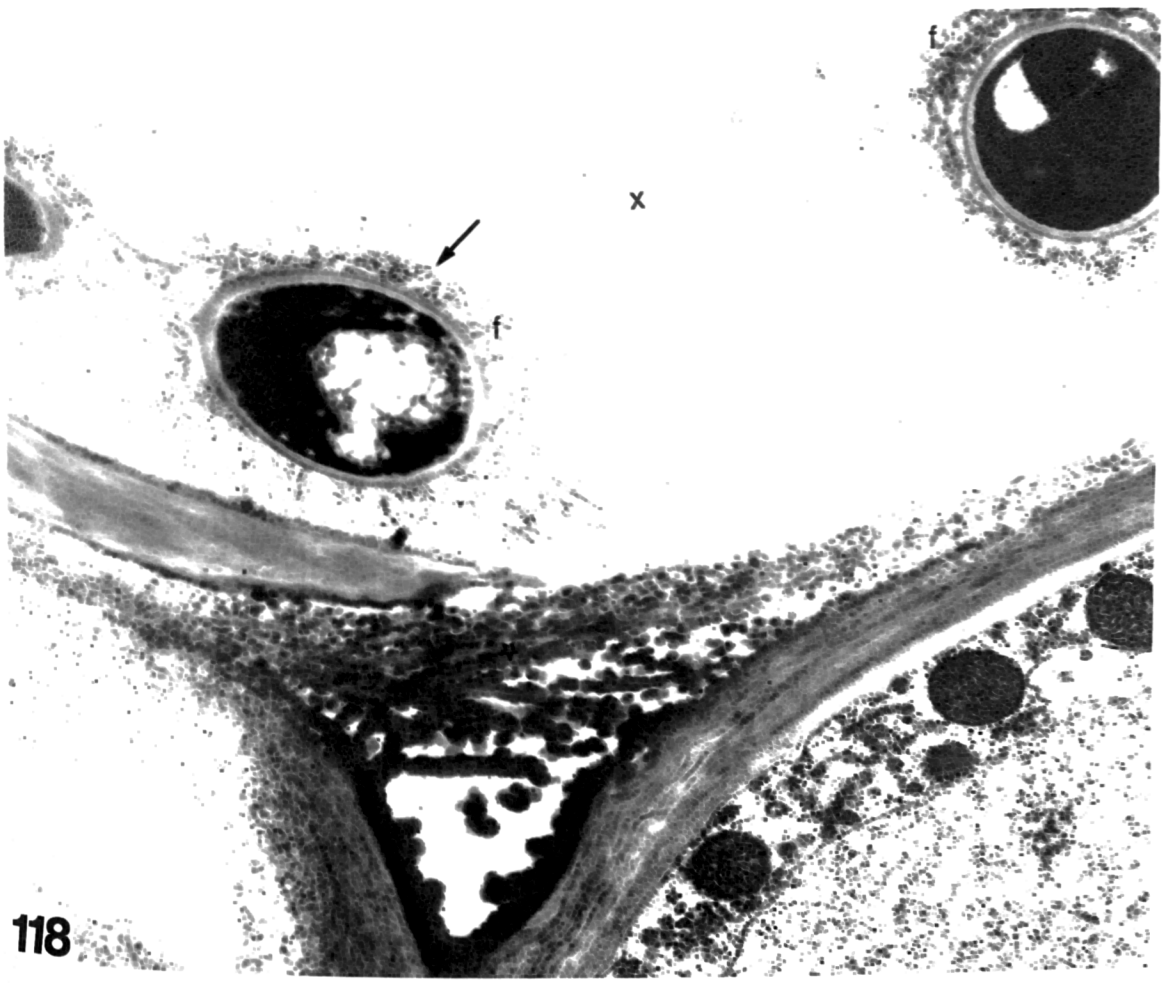

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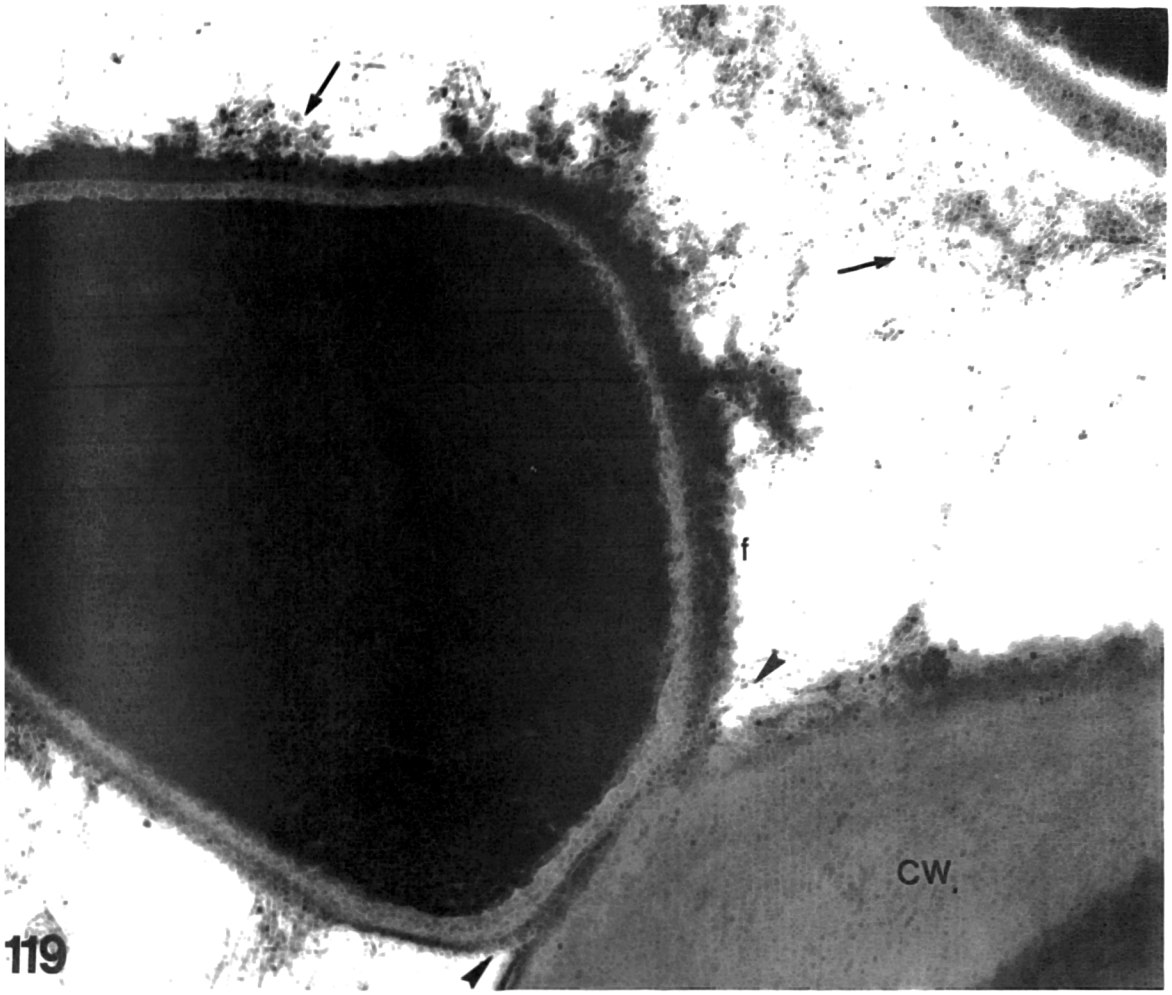
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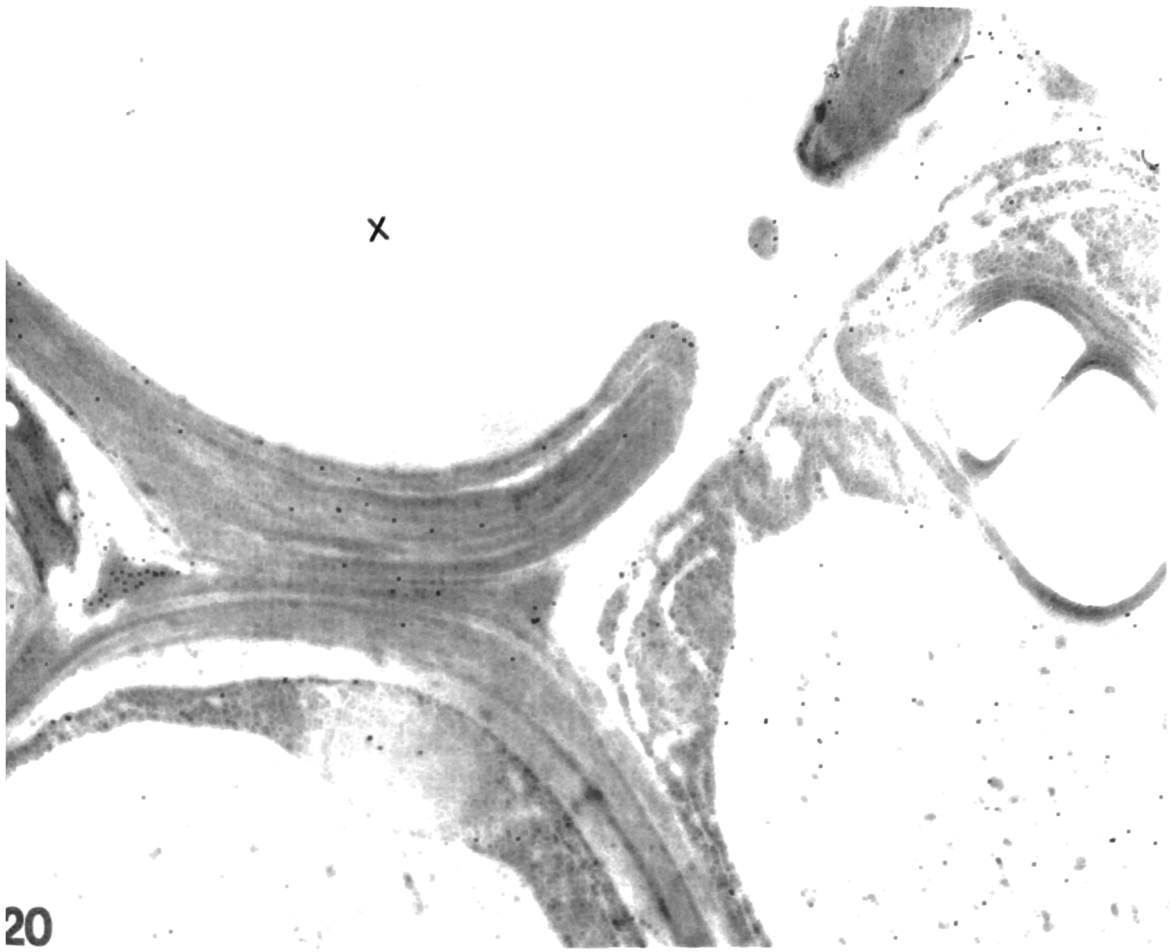
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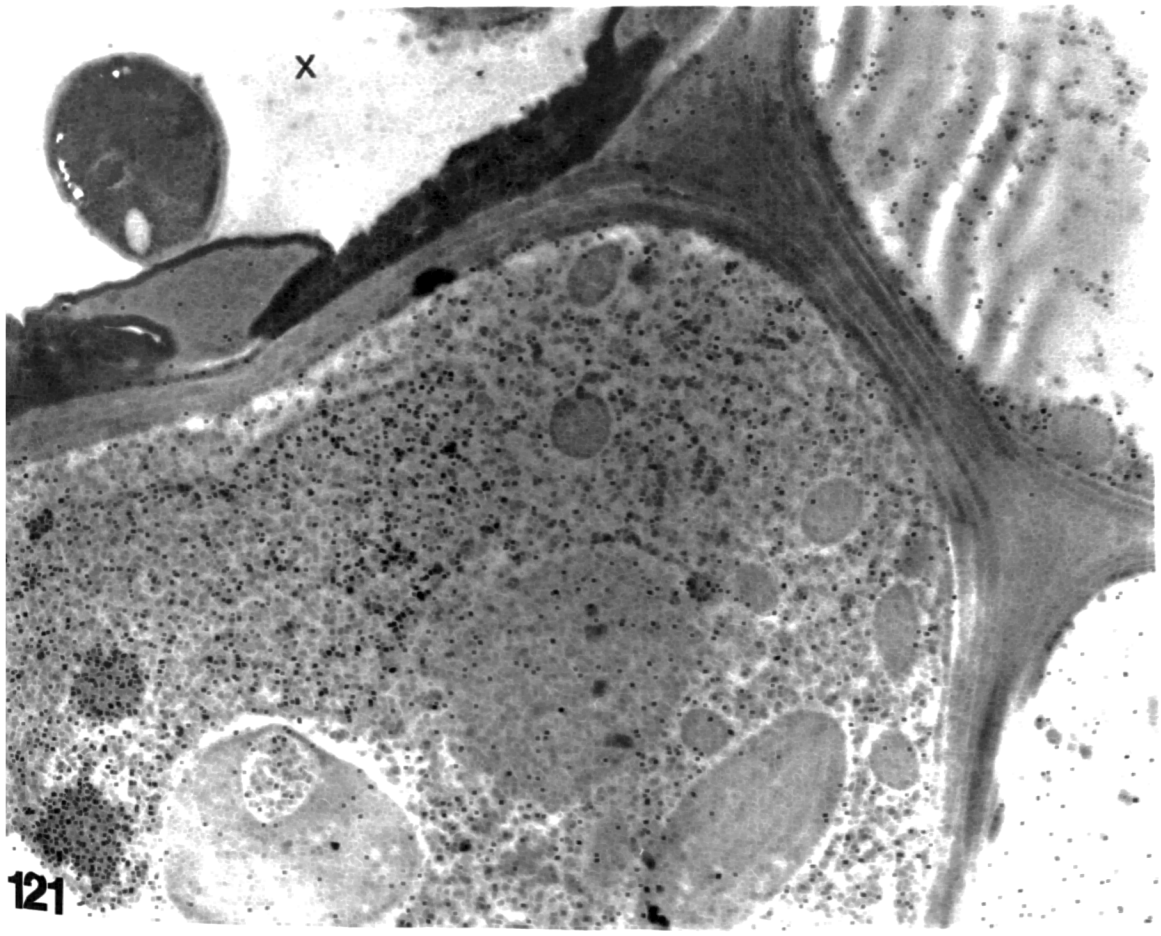
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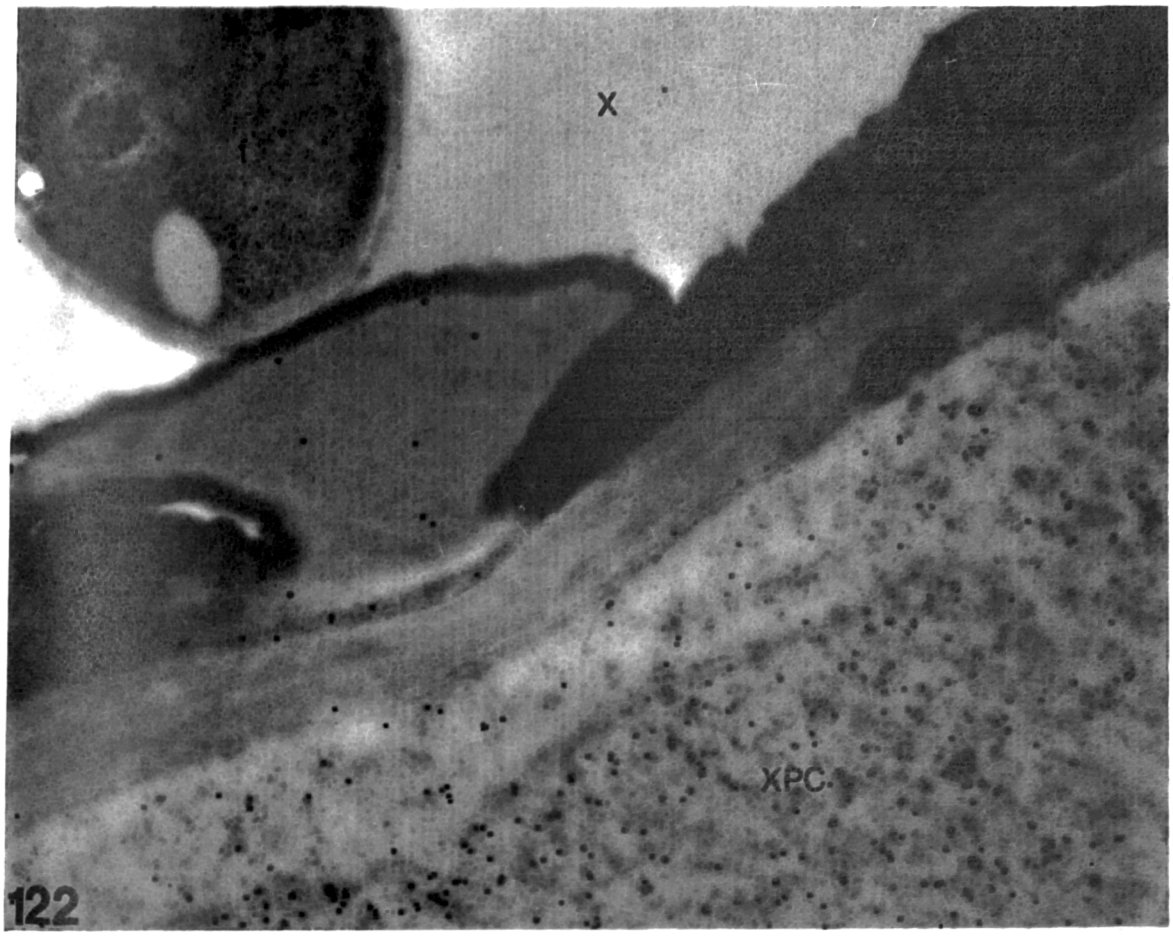
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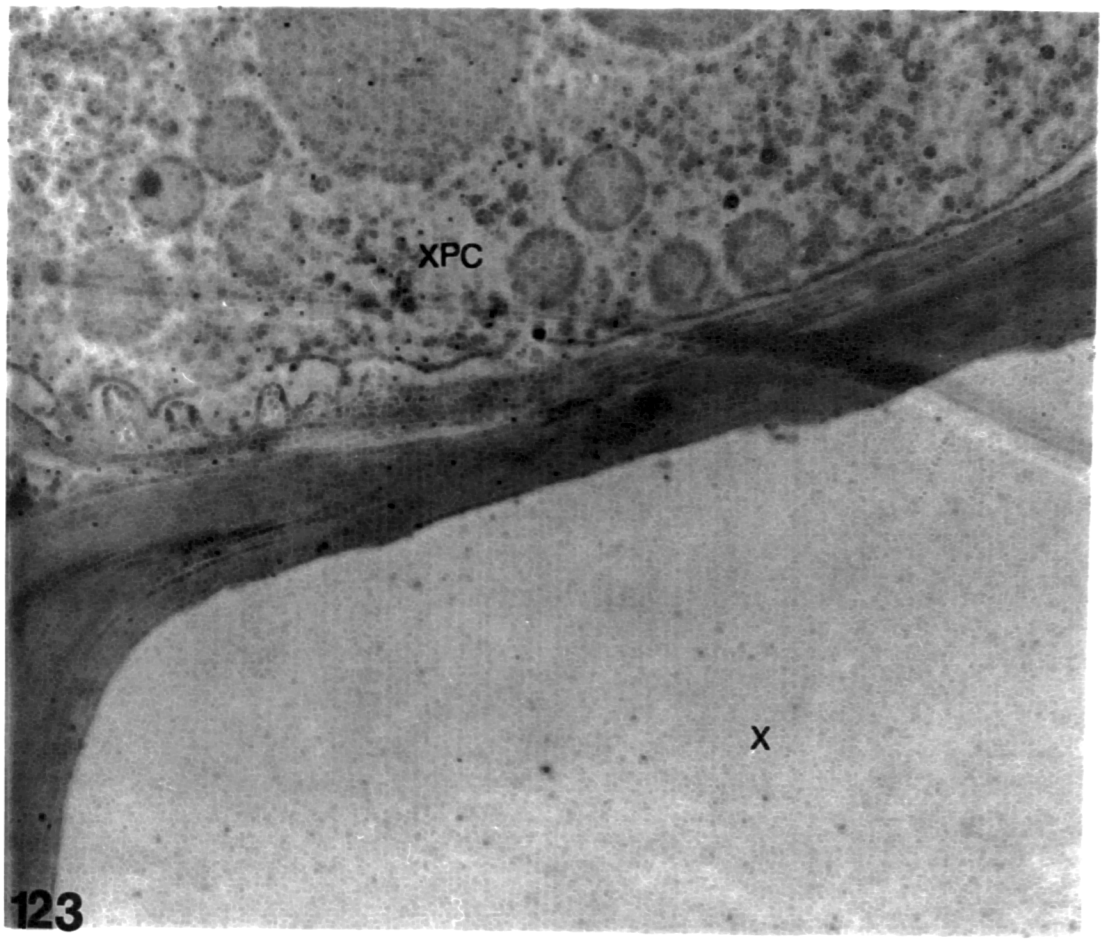
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123

3.3. High Performance Liquid Chromatography analysis (HPLC) of individual desulphoglucosinolates in OSR plants (cv. Cobra) inoculated with either a virulent isolate (161) of the pathogenic diploid strain viz.*V.d.longisporum* or an avirulent isolate (130) of a non-pathogenic haploid strain of *V.dahliae* (tomato) and of uninoculated control plants (3, 10 and 15 days after inoculation).

Two week-old OSR seedlings were inoculated by root-dipping inoculation as described in 2.4.6. with either the virulent isolate (161) or the avirulent isolate (130). Non-inoculated plants were used as controls. Twenty individual replicate plants were used for each treatment and the experiment was repeated twice. The roots, hypocotyls, cotyledons and leaves were sampled and analysed separately for each treatment. The extractions, purification, separation, identification and quantification of desulphoglucosinolates were performed as described in 2.5.4.

All data of the analysis of the individual glucosinolates from roots, hypocotyls, cotyledons and leaves are given below. For conclusions see summary Tables-3.41-,-3.42-,-3.43-,-3.44-,-3.45-,-3.46-,-3.47-, -3.48-, -3.49- and 3-50-.

3.3.1. Glucosinolates present in roots of inoculated and uninoculated OSR plants

In roots of OSR cv. Cobra, 7 glucosinolates were identified in total: one aliphatic glucosinolate: progoitrin, one aromatic: gluconasturtiin, four indole: glucobrassicin, neoglucobrassicin, 4-Methoxy-glucobrassicin and 4-OH-glucobrassicin and one unidentified glucosinolate with retention time 26.9 min. The descriptions of the glucosinolates presented in roots are given in Table-3.33- along with the retention times obtained from the standards.

Control roots (uninoculated) and roots of plants inoculated with either the virulent isolate (161) or the avirulent isolate (130) showed different profiles (i.e. a different pattern of glucosinolate production was seen) 3, 10 and 15 days after inoculation. Figure-3.36-presents the glucosinolate profiles of control roots, roots inoculated with 130 (avirulent) or 161 (the virulent isolate) for 3, 10 and 15 days after inoculation.

Neoglucobrassicin was the predominant glucosinolate in the roots of control and inoculated OSR plants at 3 days post-inoculation. At 10 and 15 days post-inoculation, neoglucobrassicin levels decreased and gluconasturtiin was the predominant glucosinolate in the roots of control plants. In roots of "130-inoculated" plants, gluconasturtiin was the predominant glucosinolate at 15 days-post inoculation. In roots of "161-inoculated" plants, neoglucobrassicin was the predominant glucosinolate 3,10 and 15 days post-inoculation.

Table-3.33- Glucosinolates found in roots of inoculated and uninoculated OSR plants (cv. Cobra)

Glucosinolate	Code	Control	Inoculated 130[1]	Inoculated 161[2]	Retention Time (min)
progoitrin	PRG	(3*,10,15)	(3,10,15)	(3,10,15)	6.65
gluconasturtiin	GLNS	(3,10,15)	(3,10,15)	(-,10,15)	20.40
glucobrassicin	GLBR	(3,15,-)	(3,10,15)	(3,10,15)	18.12
neoglucobrassicin	NGLBR	(3,10,15)	(3,10,15)	(3,10,15)	25.11
4-MeO-glucobrassicin	4-MeO-3ind	(3,-,-)	(3,10,-)	(3,10,15)	20.70
4-OH-glucobrassicin	4-OH-3ind	(-,-,15)	(-,-,15)	(-,-,-)	13.2
Unknown peak	GL-1	(-,-,15)	(-,-,15)	(-,-,15)	26.90

Table-3.34- Concentrations of glucosinolates (μmoles/gfdw) found in roots of inoculated and uninoculated OSR plants (cv. Cobra)

GLUCOSINOLATES	CONTROL			INOCULATED-130			INOCULATED-161		
Days post inoculation[3]	3	10	15	3	10	15	3	10	15
PRG	2.84~	0.325	0.213	2.45	0.294	0.4	2.08	0.38	0.14
GLNS	4.75	3.07	3.3	2.9	1.782	5.8	0	1.065	0.48
GLBR	0.8	0	0.061	1.03	0.175	0.25	1.5	0.19	0.3
NGLBR	9.3	2.04	1.285	7.9	2.014	2.229	10.4	1.119	1.295
4-MeO-3ind	1.1	0	0	1.3	0.346	0	2.2	0.225	0.6
4-OH-3ind	0	0	0.016	0	0	0.04	0	0	0
GL-1	0	0	0.005	0	0	0.018	0	0	0.03

*The numbers in brackets denote the days after inoculation. When the individual glucosinolate was detected at that particular day after inoculation the number appears in the Table. When it was not present there is a space - (undetectable amounts).

~Each value represents the mean concentration of two different extractions.

[1]an avirulent isolate of the haploid strain *V.dahliae* (tomato)

[2]a virulent isolate of the diploid strain viz.*V.d.longisporum*

[3]Plants inoculated when they were two weeks old.

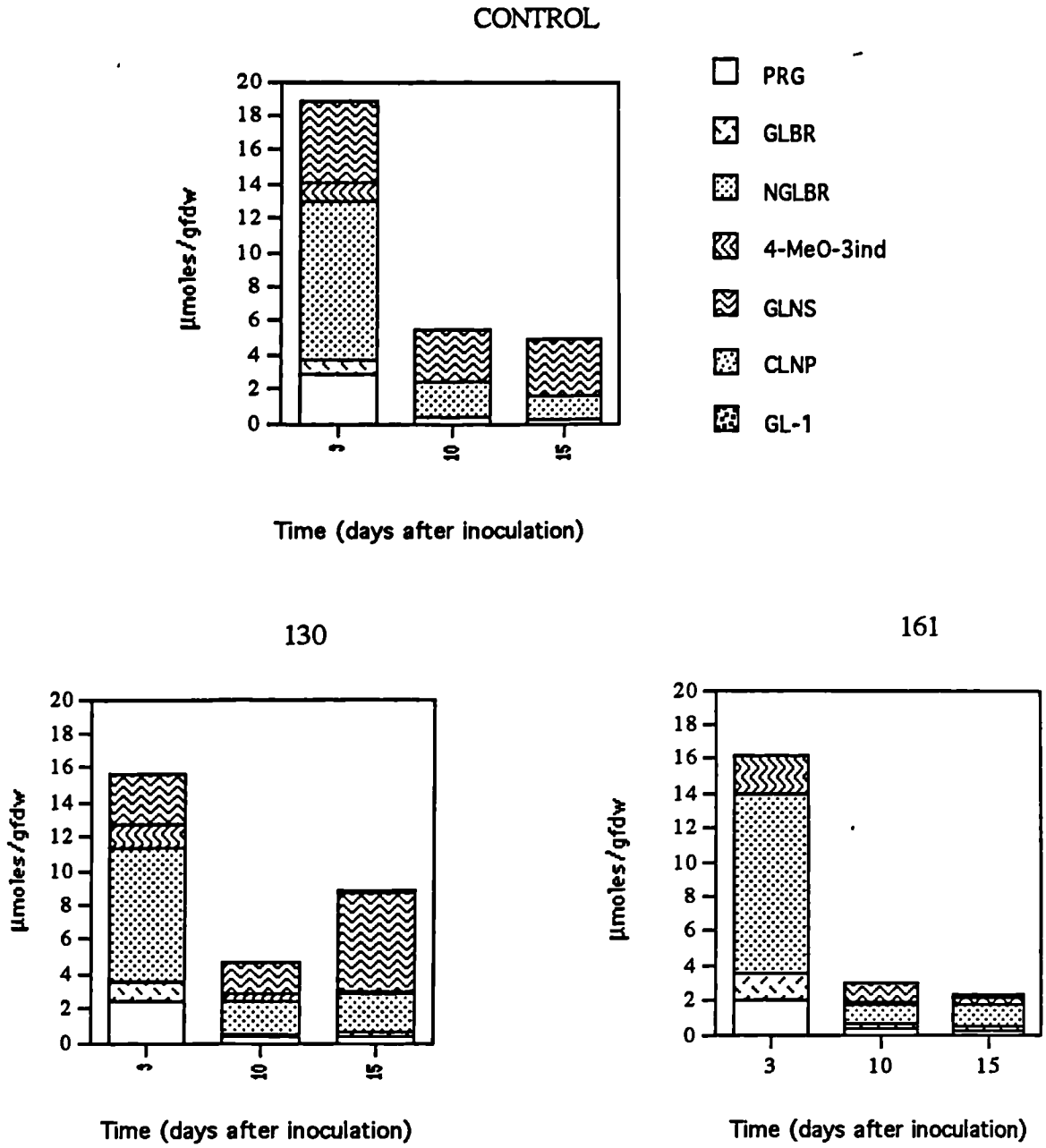
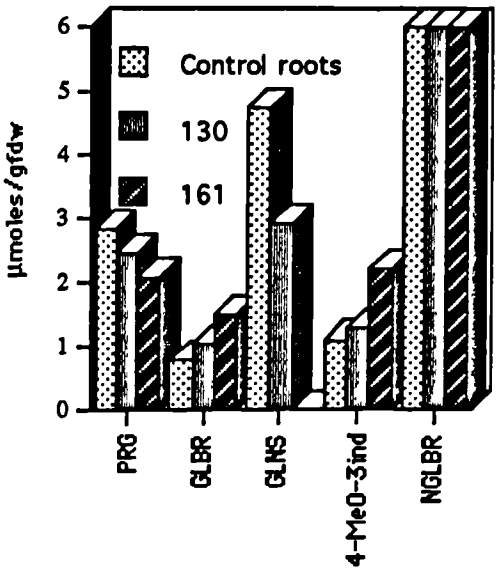
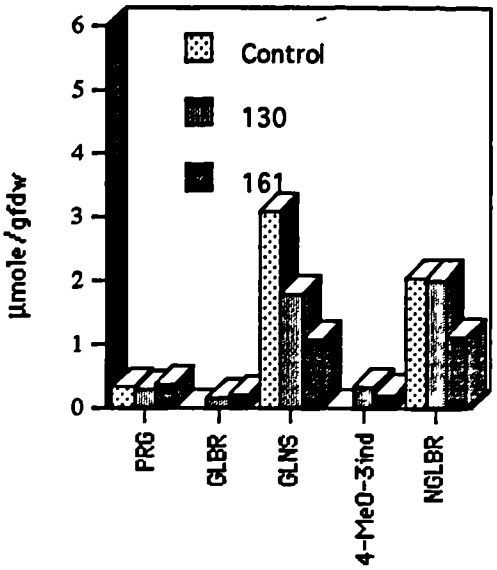


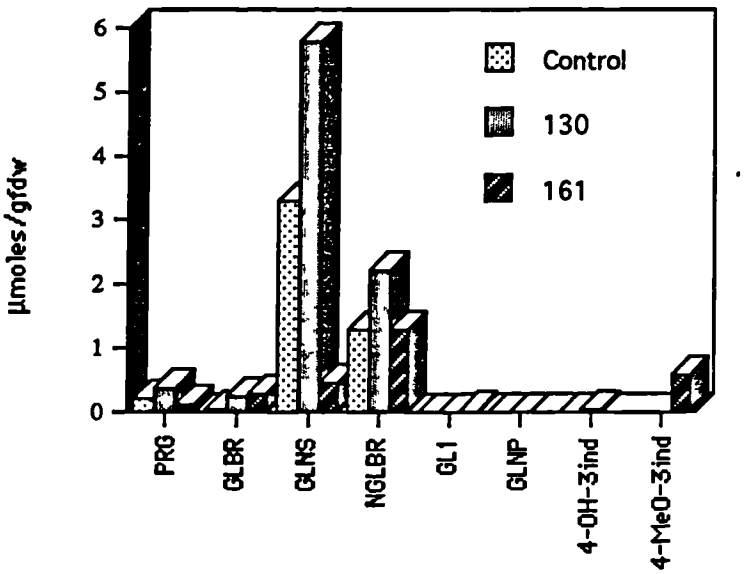
Figure-3.36- Glucosinolate profiles of roots of OSR cv. Cobra in control uninoculated plants, plants inoculated with avirulent isolate 130 of an haploid strain *V.dahliae* and plants inoculated with isolate 161 of the diploid strain viz. *V.d.longisporum*. Values represent the mean of two different extraction values (for acronyms see Table-3.33-).



Glucosinolates in roots
3 days after inoculation



Glucosinolates in roots
10 days after inoculation



Glucosinolates in roots
15 days post-inoculation

Figure-3.37- Glucosinolate content in µmoles/gfdw of roots of OSR cv. Cobra in control uninoculated plants,plants inoculated with avirulent isolate 130 of an haploid strain *V.dahliae* (tomato) and plants inoculated with isolate 161 of the diploid strain viz. *V.d.longisporum*. Each value is the mean of two different extraction values (for acronyms see Table-3.33-).

The levels of the aliphatic glucosinolate progoitrin (PRG) detected in roots of OSR cv. Cobra were higher in younger plants and decreased during growth (between 3 and 10 days post-inoculation, the level decreased almost 6 times) in uninoculated and both plants inoculated with the avirulent isolate 130 or the virulent isolate 161. Between 10 and 15 days post-inoculation, PRG remained constant. The PRG levels in control roots, inoculated with avirulent isolate 130 or with virulent isolate 161 followed the same pattern.(see figure-3.38-).

The levels of the aromatic glucosinolate gluconasturtiin (GLNS) detected in roots of OSR cv. Cobra were different among the three treatments 3, 10 and 15 days post-inoculation(see fig-3.38-). At 3 days post-inoculation, control plant roots showed the higher amounts of GLNS, followed by roots inoculated with 130. Roots inoculated with virulent 161 had undetectable levels of GLNS. At ten days post-inoculation, the amount of GLNS in roots inoculated with the avirulent isolate 130 decreased to the same levels as the roots inoculated with the virulent isolate 161. A small decrease was observed also in the control plants which continued to show the higher levels of GLNS as compared with the inoculated plants. Fifteen days after inoculation, roots of plants inoculated with the avirulent isolate 130 showed the highest amounts of GLNS as compared with the control and plants inoculated with the virulent isolate 161. Roots of the plants inoculated with the avirulent isolate 130 had almost 7 and 2 times more GLNS than the roots of plants inoculated with the virulent isolate 161 and control plants respectively.

Four indole-glucosinolates were detected in roots of control and inoculated plants (with either 130 or 161). Glucobrassicin (GLBR), neoglucobrassicin (NGLBR) and 4-Methoxy-glucobrassicin (4-MeO-3ind) showed the higher levels at 3 days post-inoculation in all three treatments. During growth, the levels of all these three indole-glucosinolates decreased (see figure-3.38-). Three days post-inoculation, plants inoculated with 161 had the higher amounts of all these three indole glucosinolates in roots as compared with controls and plants inoculated with avirulent isolate 130. Control plants showed the lower amounts of both GLBR and 4-MeO-3ind indole glucosinolates at 3 days post-inoculation. At ten days post inoculation, the amount of both these indole glucosinolates decreased almost 6 times for both inoculation treatments and in roots of control plants decreased to undetectable levels. At 15 days post-inoculation, plants inoculated with the virulent isolate 161 showed the higher amounts of 4-MeO-3ind as compared with the control and plants inoculated with the avirulent isolate 130 which had undetectable levels of this indole glucosinolate. GLBR levels in inoculated plants (with both 130, 161) were higher than the control plants 15 days post-inoculation. Neoglucobrassicin (NGLBR) was the predominant glucosinolate in the roots with high amounts 3 days post-inoculation as compared with the other glucosinolates (9.3 μmoles/gfdw, 7.88 μmoles/gfdw and 10.4 μmoles/gfdw for control uninoculated plants, those inoculated with 130 and those plants inoculated with 161 respectively). At ten days post-inoculation, NGLBR decreased for all treatments, almost 9 times. Roots of the plants inoculated with the avirulent isolate 130 showed the higher amounts of NGLBR 15 days post-inoculation as compared with control uninoculated plants and the plants inoculated with the virulent isolate 161.

The other indole glucosinolate 4-OH-glucobrassicin (4-OH-3ind) was only detected in roots in small amounts 15 days post-inoculation in the controls and plants inoculated with the avirulent isolate 130 at higher levels.

Another glucosinolate (GL-1) with retention time 26.90 was detected 15 days post-inoculation in roots for all three treatments. Plants inoculated with the virulent isolate 161 showed the higher amounts of this glucosinolate as compared with plants inoculated with the avirulent isolate 130 or the control, uninoculated plants. Roots of control plants showed the lowest amounts of this glucosinolate; there was an increase in inoculated plants(both 130,161) but the increase was higher for the roots of infected plants (ie those inoculated with 161).

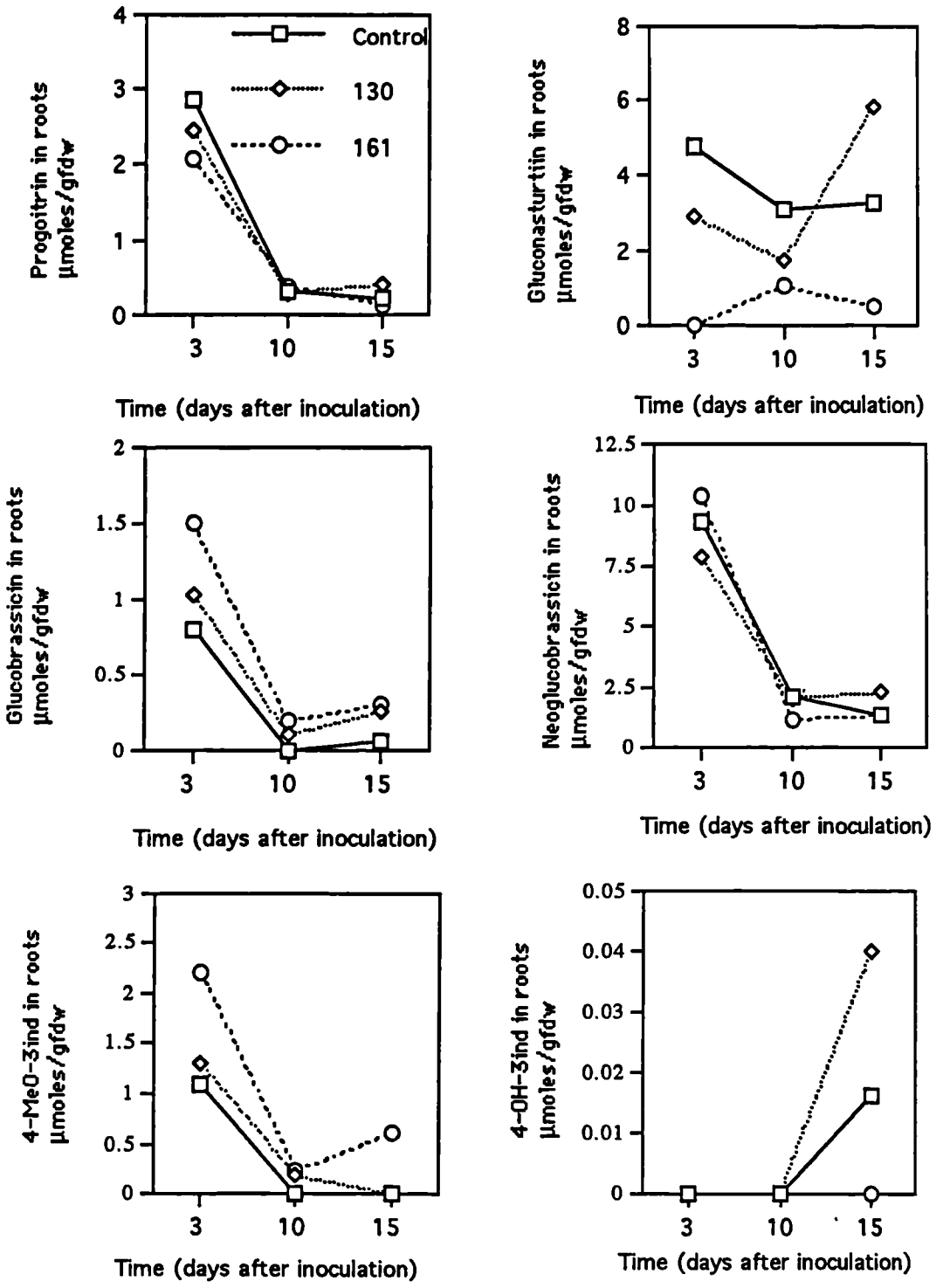


Figure-3.38- Graphic representation of the mean of duplicate determinations for PRG, GLNS, GLBR, NGLBR, 4MeO-3ind and 40H-3ind glucosinolates in the roots of control, uninoculated plants, plants inoculated with the avirulent isolate:130 of a non-pathogenic, haploid strain of *V.dahliae* (tomato) and plants inoculated with the virulent isolate: 161 of the diploid pathogenic strain viz. *V.d.longisporum* at 3, 10 and 15 days post-inoculation (for acronymous see table-3.33).

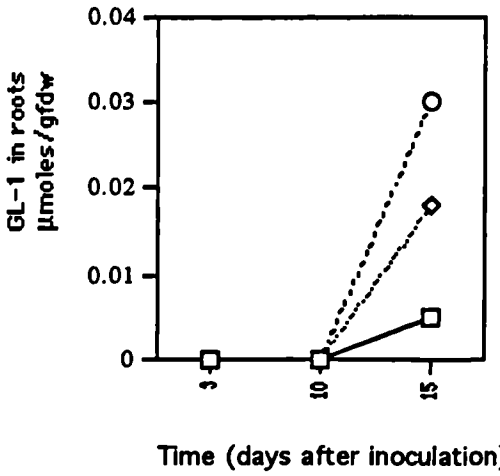


Figure-3.39- Graphic representation of GL-1(unidentified glucosinolate with retention time 26.90, see Table-3.33-) content in roots of control uninoculated plants and plants inoculated with either an avirulent isolate:130 of a non-pathogenic strain of*V.dahliae* (tomato) or a virulent isolate: 161 of the pathogenic strain viz. *V.d.longisporum.*

3.3.2. Glucosinolates present in hypocotyls of inoculated and uninoculated OSR plants (cv.Cobra).

Ten different glucosinolates were detected in hypocotyls: three aliphatic glucosinolates progoitrin (PRG), gluconapin (GLN) and napoleiferin (NPL); one aromatic gluconasturtiin (GLNS); 3 indole-glucosinolates glucobrassicin (GLBR), neoglucobrassicin (NGLBR) and 4-Methoxy-glucobrassicin (4MeO-3ind) and three others viz. GL-1 at retention time 27.98, GL-2 with retention time 15.77, and GL-5 with retention time 18.92. The descriptions of the glucosinolates presented in hypocotyls are given in Table-3.35- along with the retention times obtained from the standards.

Hypocotyls of controls and plants inoculated with either virulent isolate (161) or avirulent isolate (130) showed different profiles (i.e. different pattern of glucosinolate production was seen) 3, 10 and 15 days after inoculation. Figure-3.40- presents the glucosinolate profiles of hypocotyls of: control plants, of plants inoculated with 130 (avirulent) or with the virulent isolate 161, 3, 10 and 15 days after inoculation.

PRG and NGLBR were the predominant glucosinolates in hypocotyls 3 and 10 days post-inoculation for all three treatments (levels of PRG>levels of NGLBR at 3 days post-inoculation). Fifteen days post-inoculation, GLNS was the predominant glucosinolate in control and 130-inoculated hypocotyls (see figure -.3.42-). In "161-inoculated" hypocotyls this aromatic glucosinolate was in undetectable amounts at 15 days post-inoculation.

Table-3.35- Glucosinolates found in hypocotyls of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolate	Code	Control	Inoculated 130[1]	Inoculated 161[2]	Retention Time (min)
progoitrin	PRG	(3*,10,15)	(3,10,15)	(3,10,15)	6.65
gluconapin	GLNP	(3,10,-)	(3,10,-)	(3,10,-)	11.01
napoleiferiin	NPL	(3,-,-)	(3,-,-)	(3,-,-)	9.67
gluconasturtiin	GLNS	(-,10,15)	(-,10,15)	(-,-,10)	20.40
glucobrassicin	GLBR	(3,10,15)	(3,10,15)	(3,10,15)	18.12
neoglucobrassicin	NGLBR	(3,10,15)	(3,10,15)	(3,10,15)	25.11
4-MeO-glucobrassicin	4-MeO-3ind	(3,10,15)	(3,10,15)	(3,10,15)	20.70
Unknown peak-1	GL-1	(-,-,15)	(-,-,15)	(-,-,15)	27.98
Unknown peak-2	GL-2	(-,-,10)	(-,-,10)	(-,-,10)	15.77
Unknown peak-5	GL-5	(-,-,15)	(-,-,15)	(-,-,15)	18.92

Table-3.36- Concentrations of glucosinolates (μmoles/gfdw) found in hypocotyls of inoculated and uninoculated OSR plants (cv.Cobra).

GLUCOSINOLATES	CONTROL			INOCULATED-130			INOCULATED-161		
Days post inoculation[3]	3	10	15	3	10	15	3	10	15
PRG	7.9	2.9	0.78	6.95	2.9	1.37	8.98	1.95	0.7
GLNS	0	2.1	4.07	0	2.25	4.05	0	0.85	0
GLBR	1.39	0.6	1.2	0.92	0.9	2.3	1.17	1.34	0.9
NGLBR	5.69	2.5	2.05	4.5	3.2	3.55	5.7	1.9	1.315
4-MeO-3ind	0.57	0.17	0.17	0.3	0.37	0.86	0.28	0.45	0.4
GL-1	0	0	0.005	0	0	0.02	0	0	0.14
GL-2	0	0.1	0	0	0.7	0	0	0.1	0
GL-5	0	0	0.56	0	0	0.4	0	0	1.6
NPL	1.026	0	0	0.806	0	0	0.91	0	0
GLNP	0.31	0.3	0	0.205	0.3	0.	0.3	0.04	0

*The numbers in brackets denote the days after inoculation. When the individual glucosinolate was detected at that particular day after inoculation the number appears in the Table. When it was not present there is a space - (undetectable amounts).

[1]Avirulent isolate of an haploid strain of *V.dahliae (tomato).*

[2]Virulent isolate of the diploid strain viz. *V.d.longisporum.*

[3]Plants inoculated when they were 2 week-old.

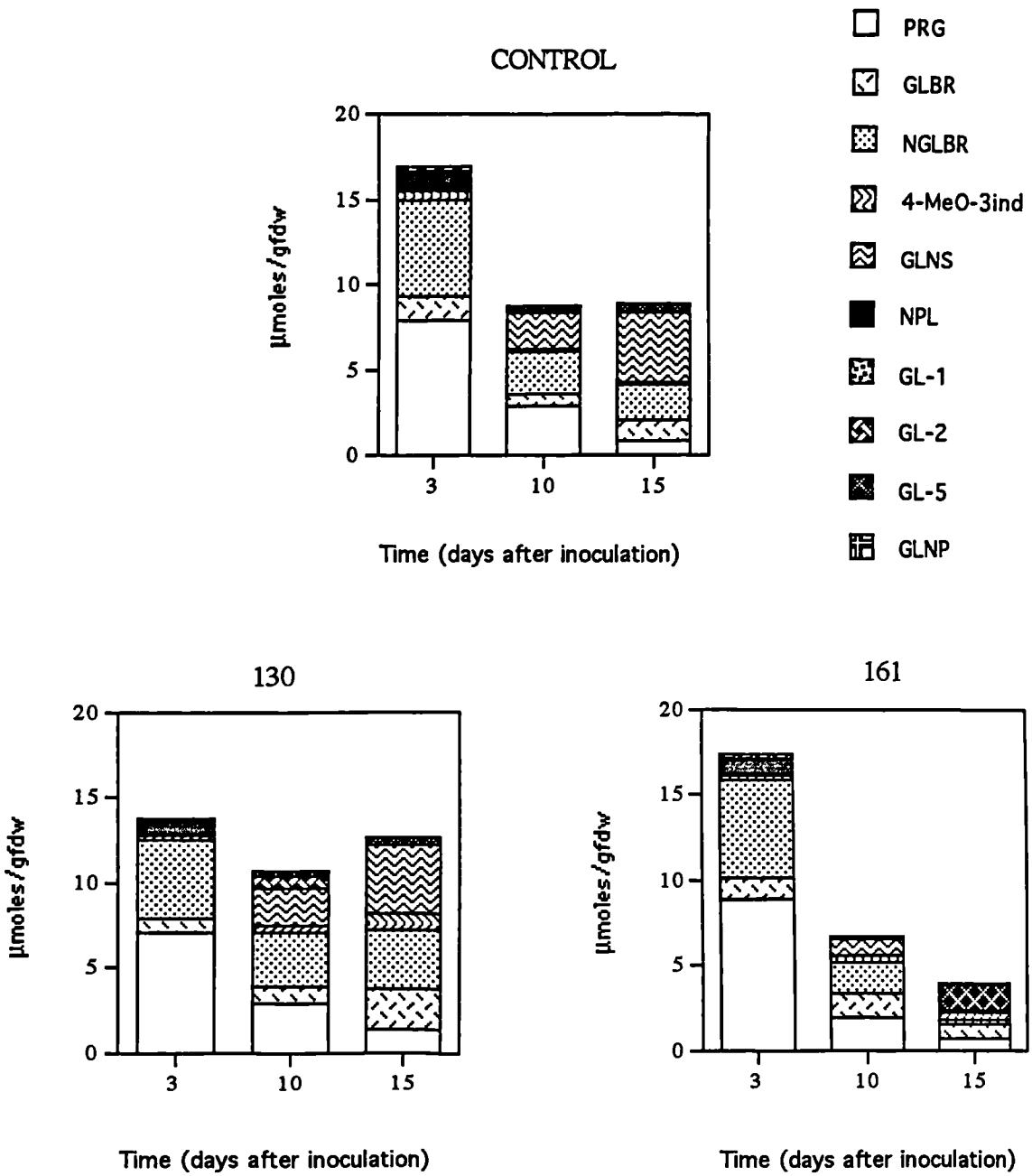
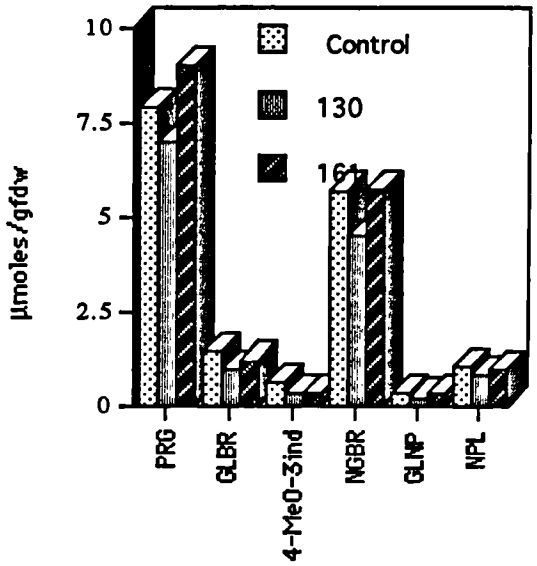
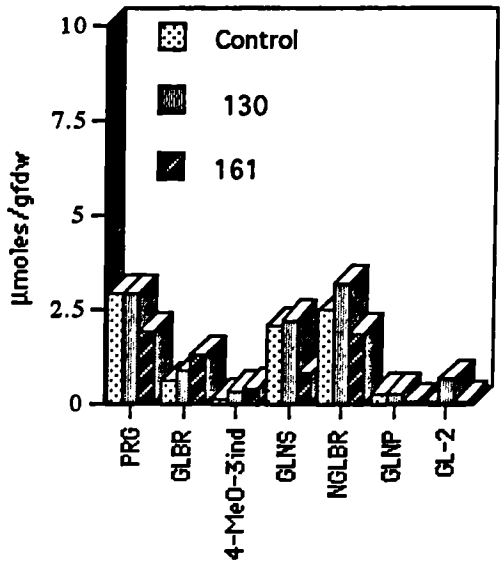


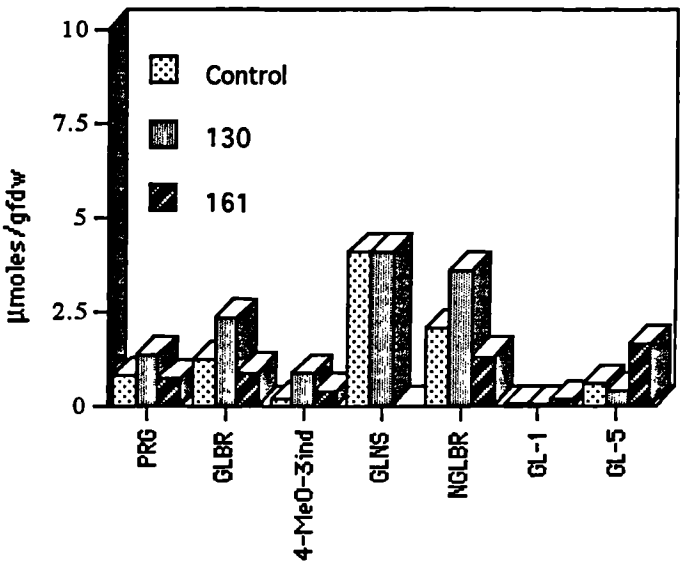
Figure-3.40- Glucosinolate profiles of hypocotyls of OSR cv. Cobra in control uninoculated plants, plants inoculated with avirulent isolate 130 of an haploid strain *V.dahliae* (tomato) and plants inoculated with isolate 161 of the diploid strain viz. *V.d.longisporum*. Values represent the mean of two different extraction values (for acronyms see Table-3.35-). Plants inoculated when they were 2 week-old.



Glucosinolates in hypocotyls
3 days after inoculation



Glucosinolates in hypocotyls
10 days after inoculation



Glucosinolates in hypocotyls
15 days after inoculation

Figure-3.41- Glucosinolate concentrations in hypocotyls of: control plants, plants inoculated with an avirulent isolate 130 of a non-pathogenic strain of *V.dahliae* and plants inoculated with the virulent isolate 161 of the diploid strain viz.*V.d.longisporum* at 3, 10 and 15 days post-inoculation (see Table-3.35- for acronyms). Plants were 2 week-old at inoculation.

Three aliphatic glucosinolates viz. PRG, GLNP and NPL were detected in hypocotyls of uninoculated plants and plants inoculated with either 130 or 161 isolates. The amounts of two of these aliphatic glucosinolates viz. PRG and NPL were decreased in hypocotyls for control plants and plants inoculated with either 130 or 161 during the time course study. The aliphatic glucosinolate GLNP found in decreased levels in hypocotyls of plants inoculated with the virulent isolate 161 as compared with levels in hypoctyls of control and '130 inoculated palnts' at 10 days post-inoculation.

PRG was detected 3, 10 and 15 days post-inoculation in hypocotyls of both uninoculated and inoculated plants.

The other aliphatic glucosinolate, GLNP, was only present in detectable amounts 3 and 10 days post-inoculation for all treatments. At ten days post-inoculation, the amount of GLNP in hypocotyls of controls and plants inoculated with the isolate 130 (avirulent) increased to the same level as the control, but decreased by almost 7.5 times in hypocotyls inoculated with the virulent isolate 161. Fifteen days post- inoculation, GLNP was not detectable in any of the 3 treatments, thus it decreased as the plants grew and matured.

NPL was detected in hypocotyls only by 3 days post-inoculation.The levels of hypocotyl NPL decreased during growth to undetectable amounts (10 and 15 days post-inoculation), see Figure-3.42-.

GLNS, the aromatic glucosinolate was detected in hypocotyls of OSR cv. Cobra 10 and 15 days post inoculation. GLNS levels increased during growth (10 and 15 days) in both controls and plants inoculated with the avirulent isolate 130. The opposite happened to the levels of GLNS in hypocotyls of plants inoculated with virulent isolate (161, virulent) where there was a slight increase by 10 days post-inoculation, but then decrease to undetectable levels at 15 days post-inoculation.

Three indole-glucosinolates were detected in hypocotyls of control and inoculated plants (using either 130 or 161) :GLBR, NGLBR and 4-MeO-3ind. The concentrations of GLBR and 4-MeO-3 ind were increased in hypocotyls of inoculated plants with either the virulent isolate, 161 or the avirulent isolate 130 at 10 days post-inoculation. At 15 days post-inoculation, the amounts of all 3 indole glucosinolates were higher in hypocotyls of plants inoculated with the avirulent isolate 130 than those inoculated with the virulent isolate 161 or control plants.

GLBR showed increased levels in hypocotyls of plants inoculated with either avirulent isolate 130, or virulent isolate 161, as compared with the levels in control hypocotyls at 10 days post-inoculation. At 15 days post-inoculation, the amount of GLBR was increased in hypocotyls of plants inoculated with avirulent isolate 130 whereas the amount in hypocotyls of plants inoculated with the virulent isolate 161 decreased to almost control levels.

The amounts of 4-MeO-3ind followed the same pattern as the amounts of GLBR. Hypocotyls of inoculated plants (with either 130 or 161) showed higher amounts at 10 days post-inoculation as compared with the control plants. At 15 days post inoculation, the amount of 4-MeO-3ind increased in hypocotyls of plants inoculated with the avirulent isolate 130. The concentrations in hypocotyls of plants inoculated with the virulent isolate 161 decreased at 15 days post-inoculation but still remained higher than the control levels.

At ten days post-inoculation, the amount of NGLBR in inoculated plants decreased to that of the control levels but at 15 days post-inoculation, the concentration of NGLBR was higher in hypocotyls of

plants inoculated with the avirulent isolate 130 as compared with those for 161 and control plants. Thus, all indole glucosinolates increased in inoculated plants with the avirulent isolate 130 at 15 days post-inoculation as compared with control or 161-inoculated plants.

Three other unknown peaks named as GL-1, GL-2 and GL-5 were detected in extracts of hypocotyls(see Table-3.35-). GL-1 showed increased levels in infected hypocotyls of plants inoculated with the virulent diploid isolate 161 of the pathogenic strain viz.*V.d.longisporum* as compared with hypocotyls of control plant and healthy plants inoculated with the avirulent isolate 130 of the haploid strain *V.dahliae*, 15 days post-inoculation. GL-2 was detected only in the control and 130-inoculated plants, 10 days post-inoculation. The amount of GL-2 was higher in the hypocotyls of inoculated plants. GL-5 was detected 15 days post-inoculation and there was an increase in levels in hypocotyls of plants inoculated with the virulent isolate 161 of the pathogenic strain viz. *V.d.longisporum* as compared with control and "130-inoculated" plants.

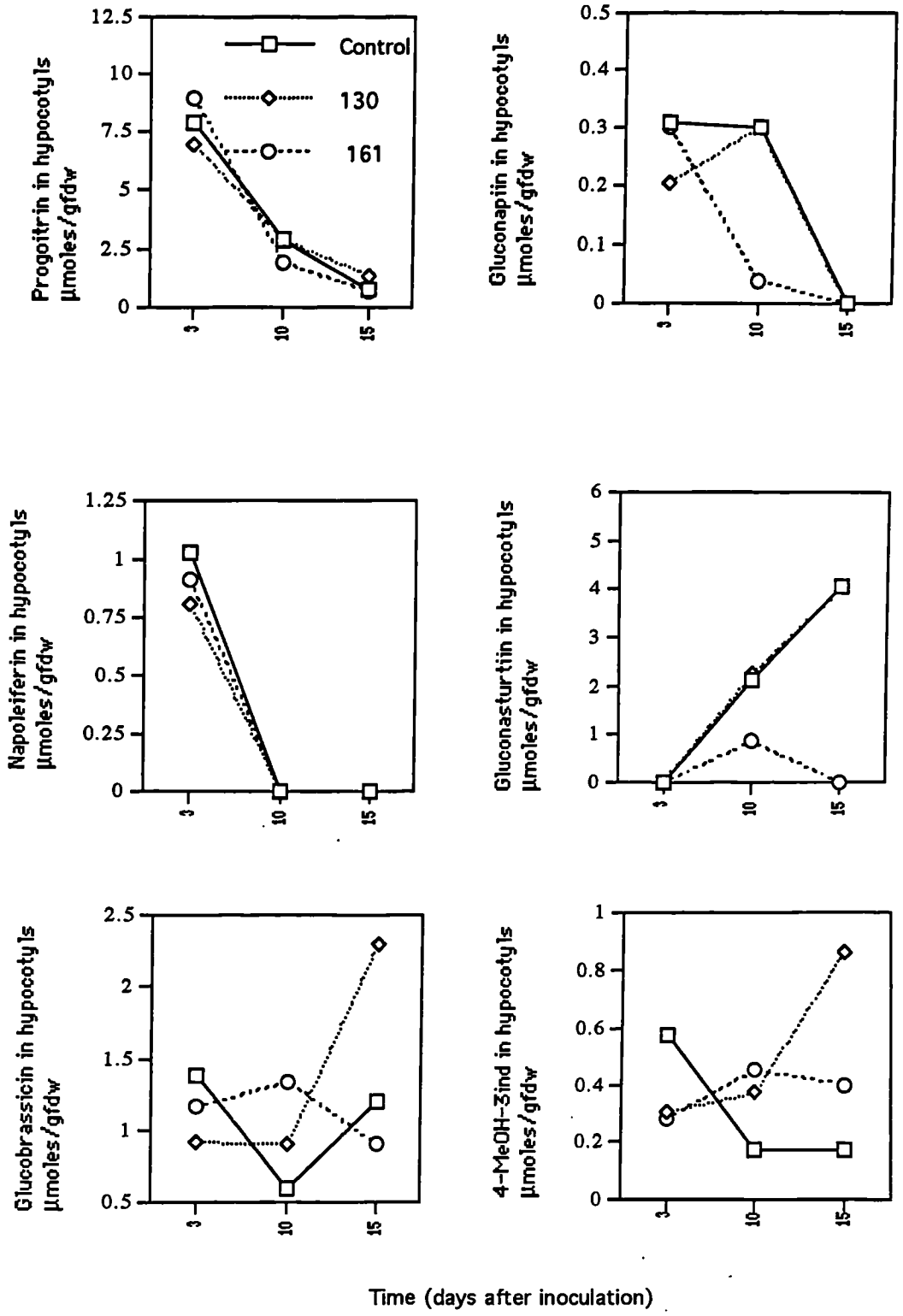


Figure-3.42- Graphic representation of the mean of duplicate determinations for PRG, GNP, NPL, GLNS, GLBR and 4-MeO-3ind, in hypocotyls of control uninoculated OSR plants (cv. Cobra), plants inoculated with an avirulent isolate 130 of a non-pathogenic haploid strain of *V.dahliae* (tomato) and plants inoculated with a virulent isolate 161 of the diploid pathogenic strain viz. *V.d.longisporum* at 3, 10 and 15 days post-inoculation (for key to glucosinolate acronyms see Table-3.35-).

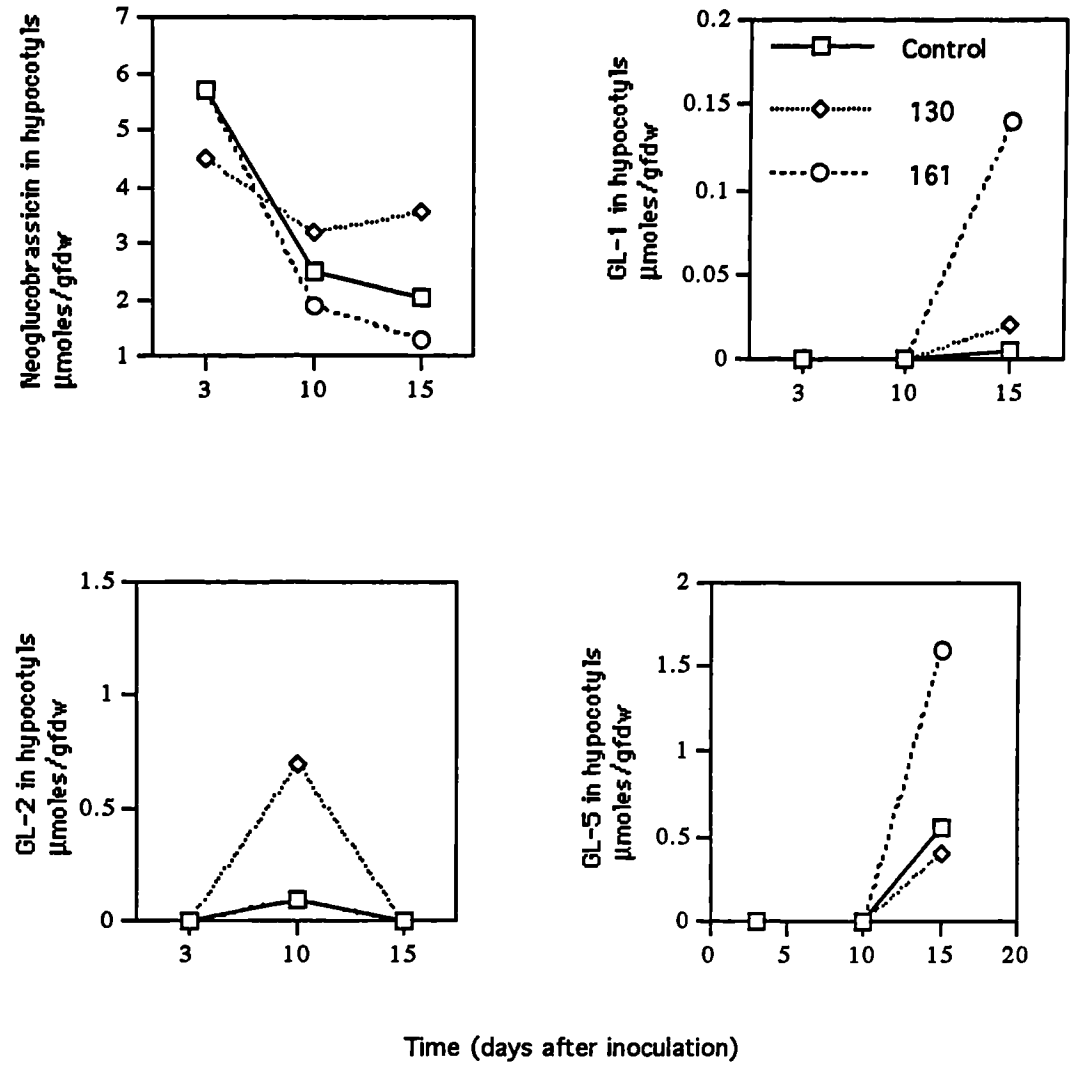


Figure-3.43- Graphic representation of the mean of duplicate determinations for NGLB, GL-1, GL-2 and GL-5 in hypocotyls of control uninoculated plants, plants inoculated with an avirulent isolate of a non-pathogenic haploid strain of *V.dahliae* (tomato) and plants inoculated with the virulent isolate 161 of the diploid pathogenic strain viz. *V.d.longisporum* at 3, 10 and 15 days post-inoculation (for key to glucosinolate acronyms see Table-3.35-). Plants were 2 week-old at inoculation.

3.3.3. Glucosinolates present in cotyledons of inoculated and uninoculated OSR plants (cv. Cobra).

In cotyledons of OSR cv. Cobra, eight glucosinolates were identified in total: two aliphatic glucosinolates: PRG and GLNP, one aromatic: GLNS, four indole: GLBR, NGLBR, 4MeO-3ind and 4OH-3ind, and one unidentified glucosinolate GL-1 with retention time 27.9 min. The descriptions of the glucosinolates presented in cotyledons are given in Table-3.37- along with the retention times obtained from standards.

Table-3.37- Glucosinolates found in cotyledons of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolate	Code	Control	Inoculated 130[1]	Inoculated 161[2]	Retention Time (min)
progoitrin	PRG	(3,*10,15)	(3,10,15)	(3,10)	6.65
gluconapin	GLNP	(-,10,15)	(3,10,15)	(-,10,15)	11.01
gluconasturtiin	GLNS	(-,10,-)	(-,10,-)	(-,10,15)	20.40
glucobrassicin	GLBR	(3,10,15)	(3,10,15)	(3,10,15)	18.12
neoglucobrassicin	NGLBR	(3,10,15)	(3,10,15)	(3,10,15)	25.11
4-MeO-glucobrassicin	4-MeO-3ind	(3,10,15)	(3,10,15)	(3,10,15)	20.70
4-OH-glucobrassicin	4-OH-3ind	(-,-,-)	(3,-,-)	(-,-,-)	13.2
Unknown peak	GL-1	(3,-,15)	(3,-,15)	(-,-,15)	27.90

Table-3.38- Concentrations of glucosinolates (µmoles/gfdw) found in cotyledons of inoculated and uninoculated OSR plants (cv.Cobra) .

GLUCOSINOLATES	CONTROL			INOCULATED-130			INOCULATED-161		
Days post inoculation[3]	3	10	15	3	10	15	3	10	15
PRG	0.605	0.63	0.096	0.99	0.48	0.25	0.403	0.63	0
GLNS	0	0.33	0	0	0.08	0	0	0.2	0.45
GLBR	1.823	1.14	2.06	2.083	1.35	3.041	1.28	4.5	0.47
NGLBR	0.145	0.26	0.17	0.045	0.04	0.25	0.025	0.13	0.107
4-MeO-3ind	0.146	0.12	0.16	0.154	0.15	0.28	0.08	0.31	0.117
4-OH-3ind	0	0	0	0.044	0	0	0	0	0
GL-1	0.06	0	0.02	0.065	0	0.01	0	0	0.009
GLNP	0	0.12	0.125	0.094	0.135	0.03	0	0.06	0.2

*The numbers in brackets denote the days after inoculation. When the individual glucosinolate was detected at that particular day after inoculation the number appears in the Table. When it was not present there is a space - (undetectable amounts).

[1]An isolate of an haploid strain of *V.dahliae (tomato).*

[2]A n isolate of the diploid strain *V.d.longisporum.*

[3]Plants were 2 week-old at inoculation.

Cotyledons of control and plants inoculated with either a virulent isolate (161) or avirulent isolate (130) showed different profiles 3, 10 and 15 days after inoculation. Figure-3.44- presents the glucosinolate

profiles of control cotyledons, cotyledons from plants inoculated with 130 (avirulent) and cotyledons from plants inoculated with the virulent isolate 161 at 3, 10 and 15 days after inoculation.

GLBR was the predominant glucosinolate in cotyledons 3, 10 and 15 days post-inoculation (see table-3.38-).

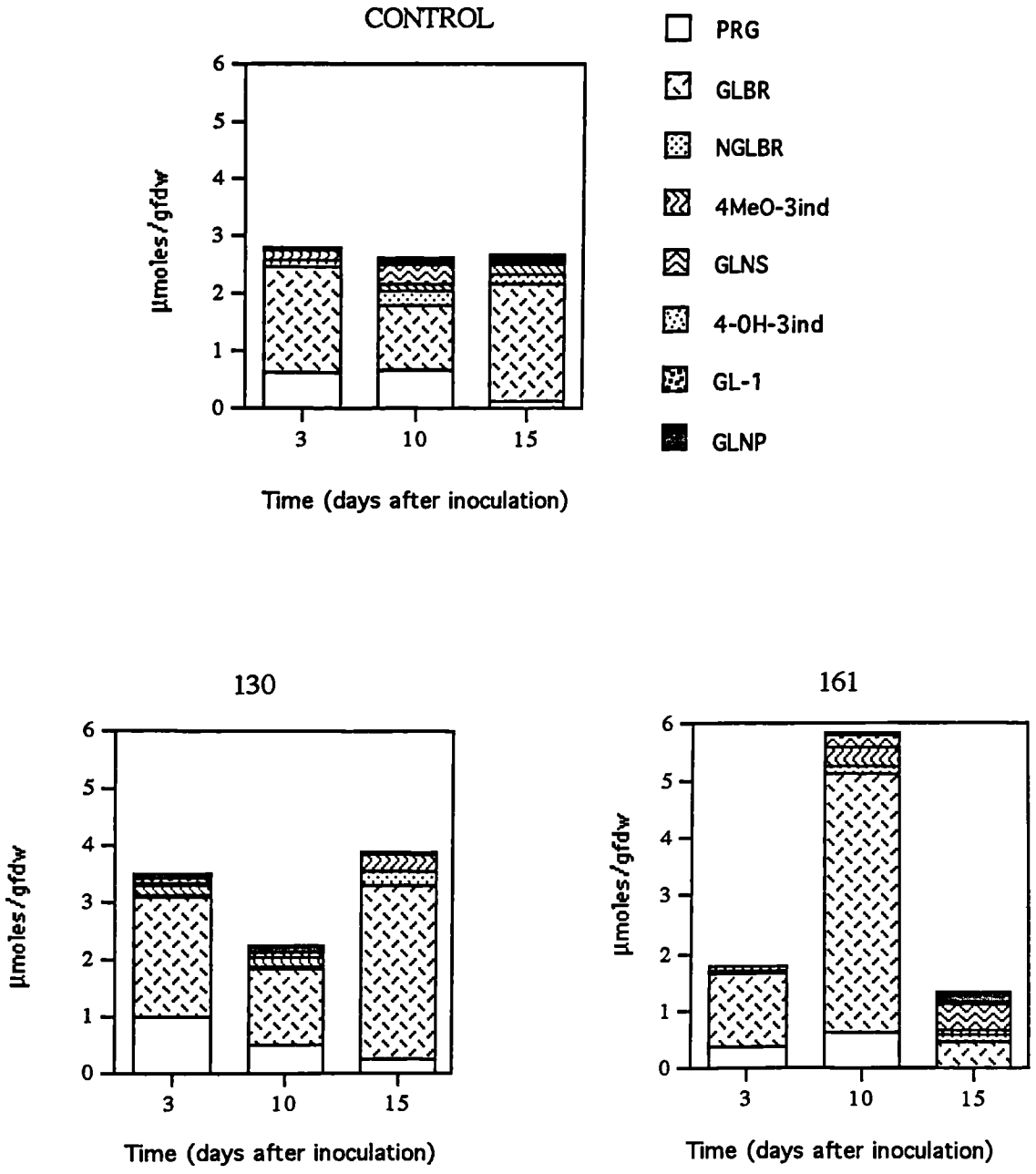


Figure-3.44- Glucosinolate profiles of cotyledons of OSR cv. Cobra in control uninoculated plants, plants inoculated with avirulent isolate 130 of an haploid strain *V.dahliae* and plants inoculated with isolate 161 of the diploid strain viz. *V.d.longisporum*. Values represent the mean of two different extraction values (for acronyms see Table-.3.37-). Plants were 2 week-old at inoculation.

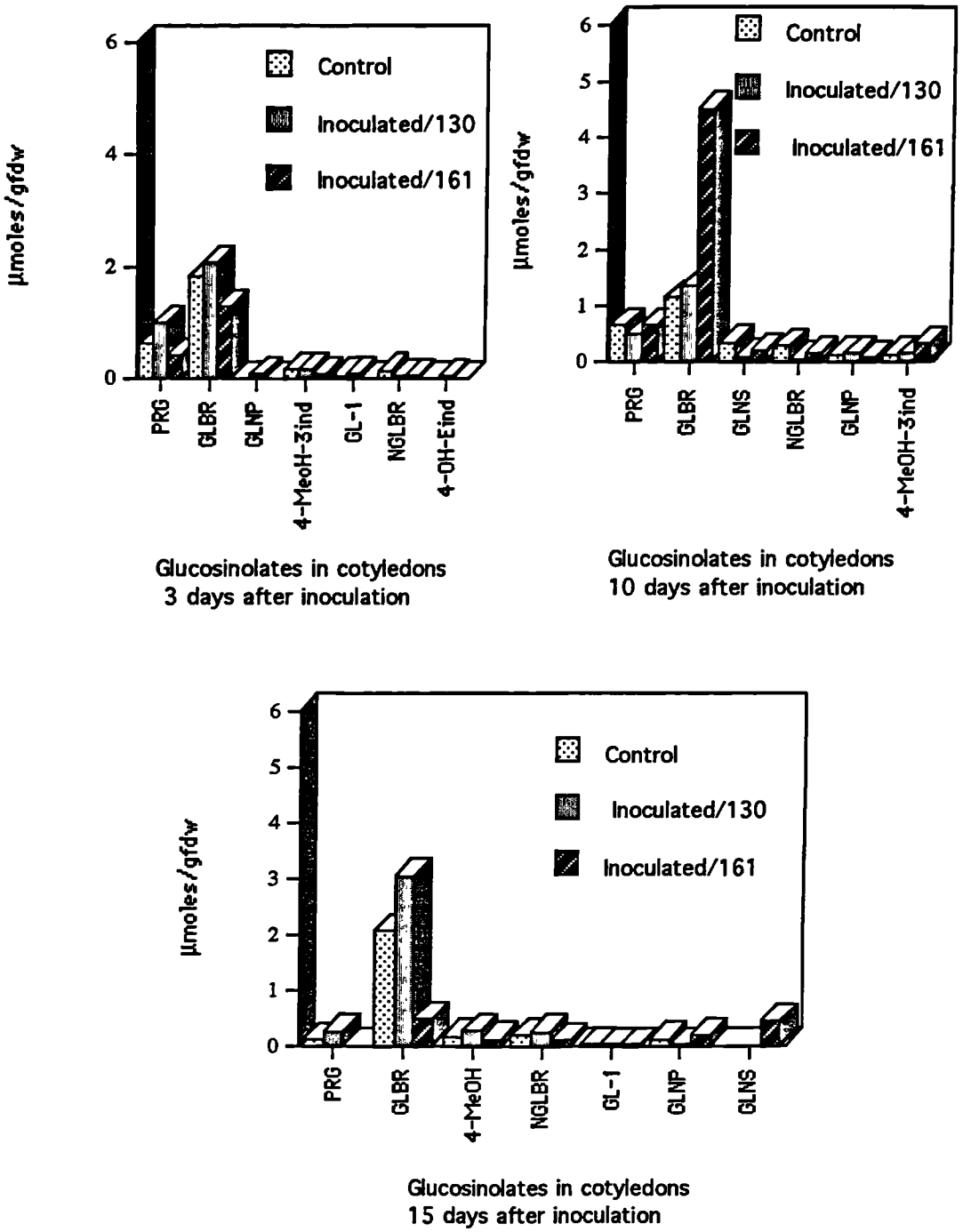


Figure-3.45- Glucosinolate content in µmoles/gfdw of cotyledons of OSR cv. Cobra in control, uninoculated plants, plants inoculated with avirulent isolate 130 of an haploid strain of *V.dahliae* (tomato) and plants inoculated with virulent isolate 161 of the diploid strain viz. *V.d.longisporum*. Each value represents the mean of two different extraction values (see Table-3.37- for key to acronyms). Plants were 2 week-old at inoculation.

Two aliphatic glucosinolates PRG and GLNP were detected in cotyledons of uninoculated plans and plants inoculated with either 130 or 161 isolates. Three days post-inoculation, the amount of both of these aliphatic glucosinolates increased in cotyledons of plants inoculated with the avirulent isolate 130 of a non-pathogenic strain of *V.dahliae.*

At 3 days post-inoculation, PRG showed the higher levels in cotyledons of plants inoculated with the avirulent isolate 130. Infected cotyledons of plants inoculated with the virulent isolate 161 had the lower amounts of PRG. During growth, the amount of PRG decreased in cotyledons for all treatments.

At 3 days post-inoculation, GLNP was only detectable in cotyledons of plants inoculated with the avirulent isolate (130). At ten days post-inoculation, the concentration of GLNP increased in all treatments, while cotyledons of plants inoculated with the avirulent isolate 130 still showed the highest levels. Cotyledons of the plants inoculated with the virulent isolate 161 showed the lower levels of GLNP. At 15 days post-inoculation, the amount of GLNP was still increased in cotyledons in both of control and plants inoculated with 161, but decreased markebly in the cotyledons of those plants inoculated with the avirulent isolate (130).

The aromatic glucosinolate GLNS was detected in cotyledons of OSR cv. Cobra at 10 days post-inoculation for all treatments. Control cotyledons showed the highest amount, followed by the cotyledons of plants inoculated with the avirulent isolate 130 and infected cotyledons of plants inoculated with the virulent isolate 161. At 15 days post-inoculation, the amount of GLNS decreased to undetectable levels for both control and plants inoculated with 130 but it increased markedly in infected cotyledons of plants inoculated with the virulent isolate 161.

Four indole-glucosinolates were detected in cotyledons: GLBR, NGLBR, 4-MeO-3ind and 4OH-3ind. GLBR and 4-MeO-3ind followed the same pattern (see figures -3.46-, -3.47-) during the time course study. Cotyledons of the control and plants inoculated with the avirulent isolate 130 showed a slight reduction in the amounts of these two glucosinolates 3 to 10 days post-inoculation, followed by an increase 15 days post-inoculation. The amount of these two glucosinolates was higher for cotyledons inoculated with 130 as compared with those for the controls for all time periods post-inoculation, reaching the maximum difference at 15 days post-inoculation. On the contrary, cotyledons of plants inoculated with the virulent isolate 161 showed the lower levels of these two indole-glucosinolates as compared with the control plants 3 and 15 days post-inoculation. At 10 days post-inoculation, when yellow (chlorotic) cotyledons started to become brown (necrotic) in plants inoculated with 161, the amount of these two indole-glucosinolates increased markedly reaching almost double the levels of cotyledons of control uninoculated plants and healthy cotyledons of the plants inoculated with the avirulent isolate 130.

NGLBR followed a different pattern; its levels in cotyledons of controls and those of plants inoculated with the virulent isolate 161 increased at first and then decreased at 15 days post-inoculation (control plants showed the highest amount of this glucosinolate in cotyledons). In the cotyledons of plants inoculated with the avirulent isolate 130, there was a marked increase 15 days post-inoculation, as compared with the amounts of this glucosinolate 3 and 10 days post-inoculation.

4-OH-3ind glucosinolate was detected only in the cotyledons of the 130-inoculated plants at 3 days post-inoculation.

GL-1 was undetectable in cotyledons of plants inoculated with the virulent isolate 161 at 3 days post-inoculation. Cotyledons of controls and those of 130-inoculated plants showed the similar amounts of this glucosinolate. At 15 days post-inoculation, almost the same amounts of GL-1 were detected in all three treatments .

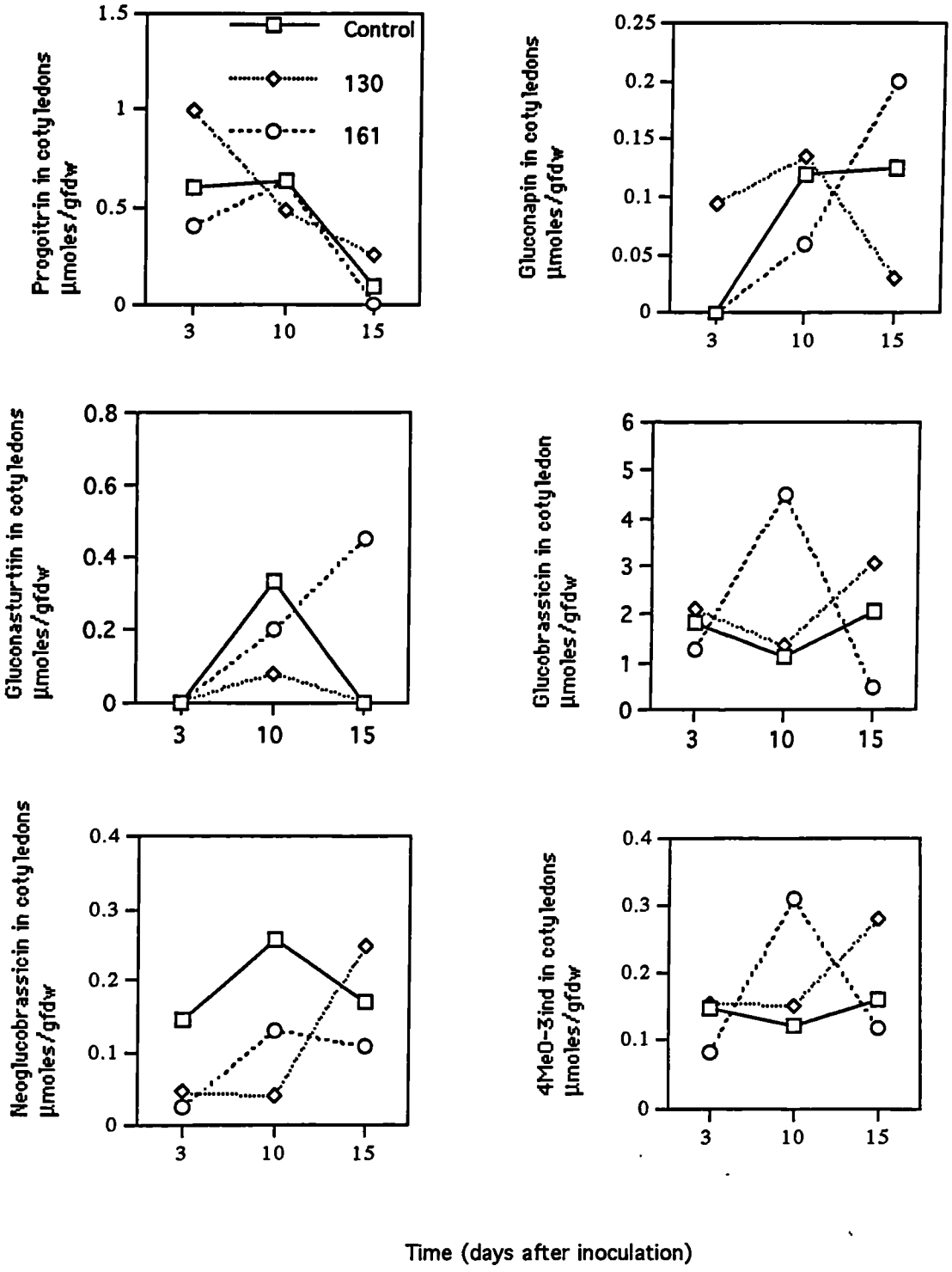


Fig-3.46- Graphic representation of the mean of duplicate determinations for PRG, GLNP, GLNS, GLBR, NGLBR and 4-MeO-3ind, in cotyledons of control uninoculated plants, plants inoculated with an avirulent isolate of a non-pathogenic haploid strain of*V.dahliae* and plants inoculated with a virulent isolate 161 of the diploid pathogenic strain viz. *V.d.longisporum* at 3, 10 and 15 days post-inoculation (for glucosinolate acronyms see Table-3.37-). Plants were 2 week-old at inoculation.

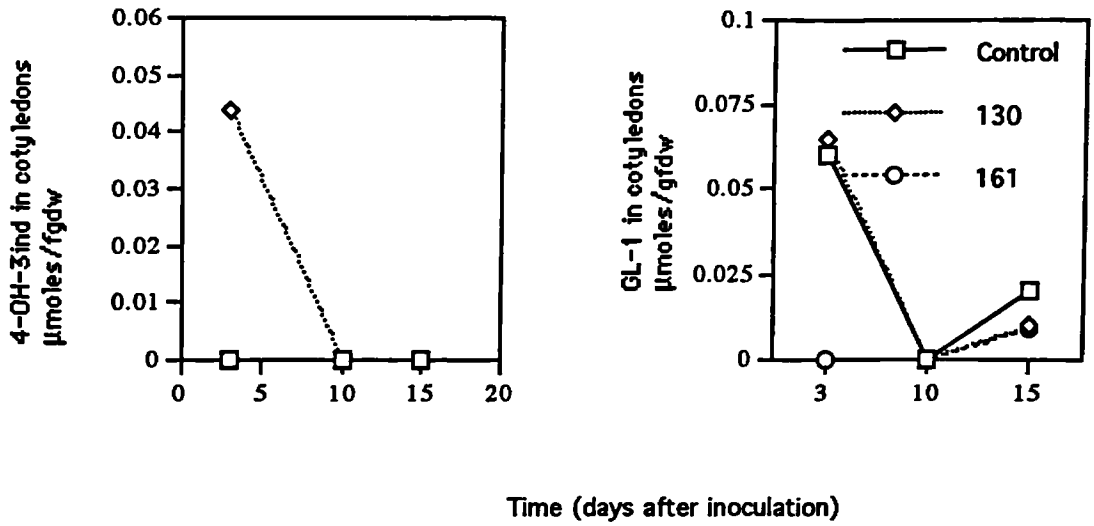


Figure-3.47- Graphic representation of the mean of duplicate determinations for 4-OH 3ind and GL-1, in cotyledons of control uninoculated plants, plants inoculated with an avirulent isolate of a non-pathogenic haploid strain of *V.dahliae* and plants inoculated with a virulent isolate 161 of the diploid pathogenic strain viz *V.d.longisporum* 3, 10 and 15 days post-inoculation (for glucosinolate acronyms see Table -3.37-). Plants were 2 week-old at inoculation.

3.3.4. Glucosinolates present in leaves of inoculated and uninoculated OSR plants (cv. Cobra).

In leaves of OSR cv. Cobra, 11 glucosinolates were identified in total: two aliphatic glucosinolates: PRG and GLNP, one aromatic: GLNS, four indole: GLBR, NGLBR, 4MeO-3ind and 4-OH-3ind and four unidentified glucosinolates (GL-1, GL-2, GL-3 and GL-4) with retention times 26.9 ,15.82, 9.25 and 9.53 min. The descriptions of the glucosinolates presented in leaves are given in Table-3.39- along with the retention times obtained from the standards.

Leaves of controls and plants inoculated with either the virulent isolate (161) or avirulent isolate (130) showed different profiles 3, 10 and 15 days after inoculation. Figure-3.48-presents the glucosinolate profiles of leaves of control plants,of plants inoculated with avirulent isolate 130 and of plants inoculated with the virulent isolate 161; 3, 10 and 15 days after inoculation.

GLBR and PRG were the predominant glucosinolates in control and inoculated OSR plants in the leaves 3, 10 and 15 days post-inoculation (see table-3.40-).

Table-3.39- Glucosinolates found in leaves of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolate	Code	Control	Inoculated 130[1]	Inoculated 161[2]	Retention Time (min)
progoitrin	PRG	(3*,10,15)	(3,10,15)	(3,10,15)	6.65
gluconasturtiin	GLNS	(10,15)	(10,15)	(10,15)	20.40
gluconapiin	GLNP	(3,10,15)	(3,-,15)	(3,10,15)	
glucobrassicin	GLBR	(3,10,15)	(3,10,15)	(3,10,15)	18.12
neoglucobrassicin	NGLBR	(3,10,-)	(3,10,-)	(3,10,-)	25.11
4-MeO-glucobrassicin	4-MeO-3ind	(3,10,15)	(3,10,15)	(3,10)	20.70
4-OH-glucobrassicin	4-OH-3ind	(3,-,15)	(3,-,-)	(3,-,15)	13.2
Unknown peak	GL-1	(-,-,15)	(-,-,15)	(-,-,15)	26.90
Unknown peak	GL-2	(-,-,-)	(-,-,-)	(-,10,-)	15.82
Unknown peak	GL-3	(3,10,-)	(3,10,-)	(-,-,-)	9.25
Unknown peak	GL-4	(3,10,-)	(3,10,-)	(-,-,-)	9.53

Table-3.40- Concentrations of glucosinolates (μmoles/gfdw) found in leaves of inoculated and uninoculated OSR plants (cv. Cobra).

GLUCOSINOLATES	CONTROL			INOCULATED-130			INOCULATED-161		
Days post inoculation[3]	3	10	15	3	10	15	3	10	15
PRG	2.95	0.95	0.72	2.7	4.3	1.1	2.36	1.45	0.43
GLNS	0	0.2	0.38	0	0.077	0.62	0	0.5	1.12
GLBR	4.42	2.6	4.22	5.4	1.43	4.38	4.52	3.1	2.42
NGLBR	0.185	0.17	0.274	0.27	0.04	0.223	0.2	0.18	0.271
4-MeO-3ind	0.035	0.04	0.086	0.05	0.006	0.086	0.05	0.107	0
4-OH-3ind	0.088	0	0.002	0.02	0	0.001	0.013	0	0.207
GL-1	0	0	0.007	0	0	0.009	0	0	0.01
GL-2	0	0	0	0	0.03	0	.0	0.082	0
GL-3	0	0	0.16	0	0	0.205	0	0	0
GL-4	0	0	0.21	0	0	0.37	0	0	0
GLNP	0.35	0.04	0.02	0.27	0	0.047	0.215	0.06	0

*The numbers in brackets denote the days after inoculation. When the individual glucosinolate was detected at that particular day after inoculation the number appears in the Table. When it was not present there is a space - (undetectable amounts).

[1] An isolate of a haploid strain of *V.dahliae* (tomato).

[2] An isolate of the diploid strain viz. *V.d.longisporum.*

[3]Plants were 2 week-old at inoculation.

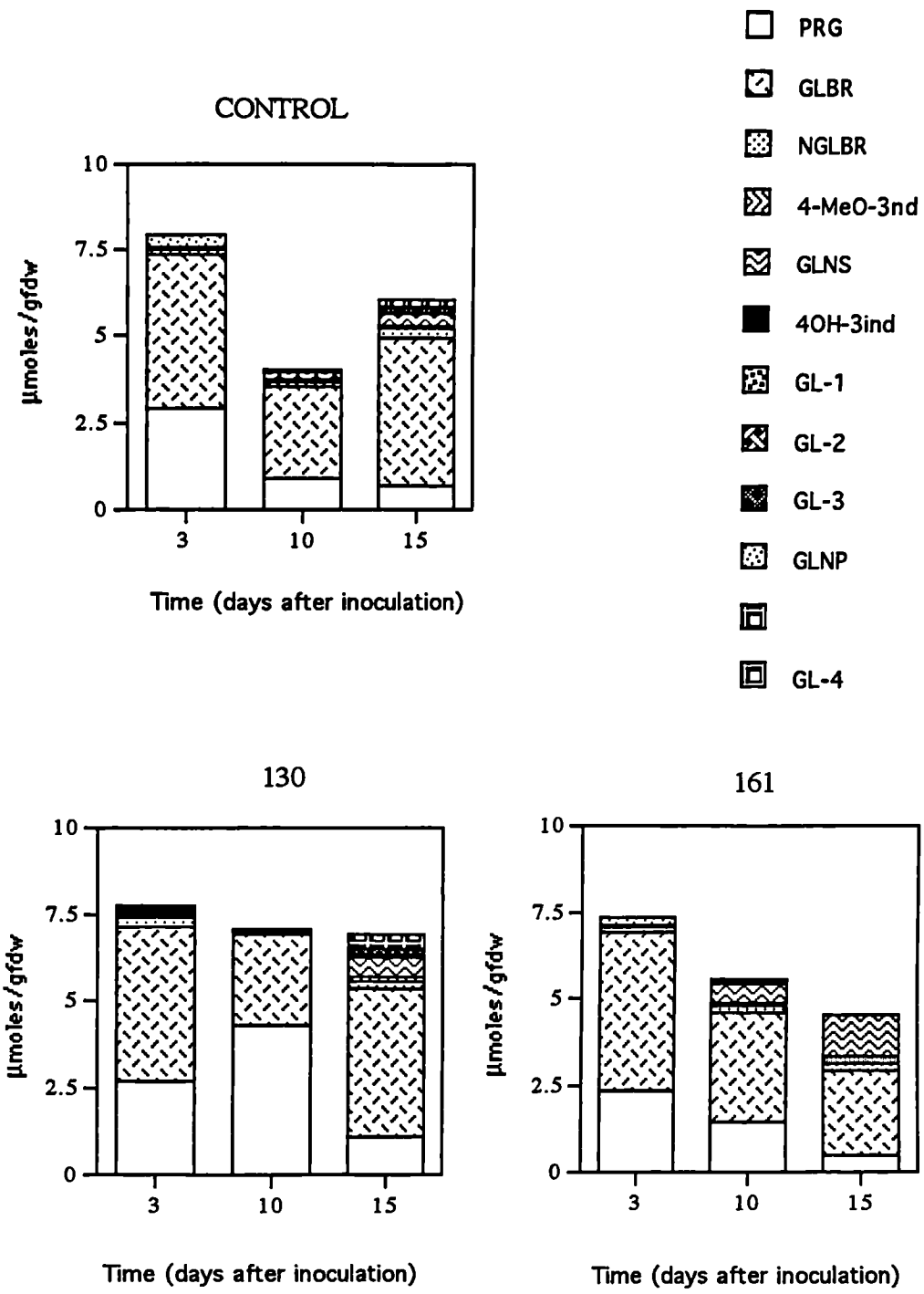
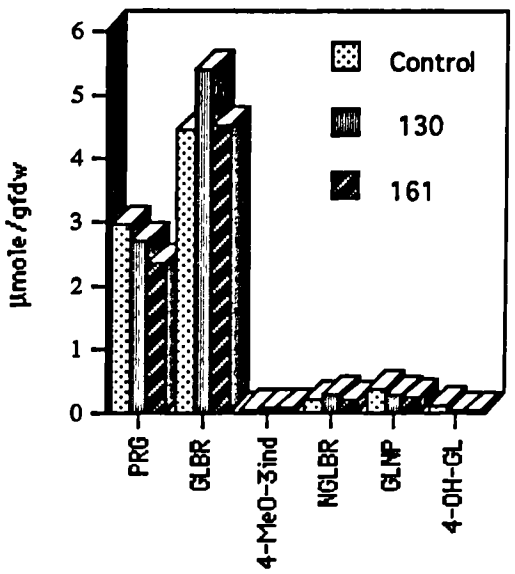
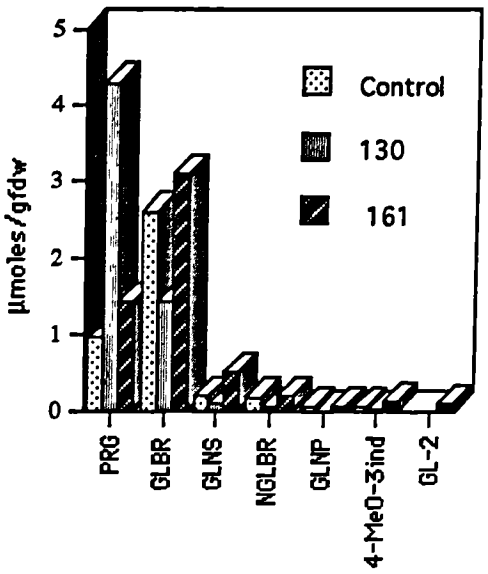


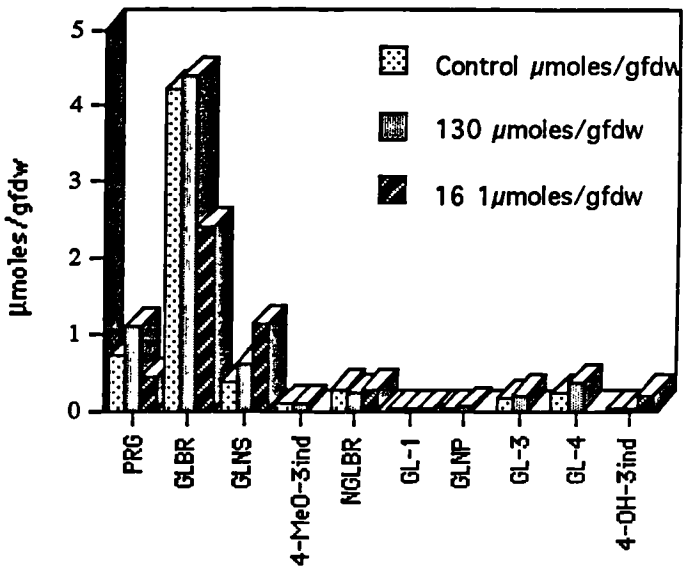
Figure-3.48- Glucosinolate profiles of leaves of OSR cv. Cobra in control uninoculated plants, plants inoculated with avirulent isolate 130 of an haploid strain *V.dahliae* (tomato) and plants inoculated with isolate 161 of the diploid strain viz. *V.d.longisporum*. Values represent the mean of two different extraction values (for acronyms see Table-3.39-). Plants were 2 week-old at inoculation.



Glucosinolates in leaves
3 days after inoculation



Glucosinolates in leaves
10 days after inoculation



Glucosinolates in leaves
15 days after inoculation

Figure-3.49- Glucosinolate content in µmoles/gfdw in leaves of OSR cv. Cobra of control uninoculated plants, plants inoculated with an avirulent isolate 130 of a haploid strain of *V.dahliae* and plants inoculated with a virulent isolate 161 of the diploid strain viz. *V.d.longisporum*. Values represent the mean of two different extraction values (see Table-3.39- for glucosinolate acronyms). Plants were 2 week-old at inoculation.

Two aliphatic glucosinolates PRG and GLNP were detected in leaves from all treatments. The amount of PRG was decreased in leaves during the time of the study in both control plants and in plants inoculated with the virulent isolate 161 of the pathogenic diploid strain viz.*V.d.longisporum*. On the contrary, the concentration of this aliphatic glucosinolate increased markedly in leaves of plants inoculated with the avirulent isolate 130 of the non-pathogenic strain of *V.dahliae*, 10 days post-inoculation; at 15 days post-inoculation the amount of PRG had decreased to control levels. GLNP followed the same pattern in all treatments, decreasing markedly between 3 and 10 days post-inoculation and then remaining constant between 10 and 15 days post-inoculation (see figure-3.50)

The aromatic glucosinolate GLNS was detected in leaves at 10 and 15 days post-inoculation. It increased over time for all treatments but leaves of plants inoculated with the virulent isolate 161 showed the highest amounts of this glucosinolate (see figure-3.50)

Four indole-glucosinolates were detected in leaves : GLBR, NGLBR, 4-MeO-3ind and 4-OH-3ind. GLBR was found at increased levels 3 days post-inoculation followed by a decrease at 10 days post-inoculation for all treatments, its levels increased again 15 days post-inoculation only for leaves of the control plants and those of plants inoculated with the avirulent isolate 130. Leaves of infected plants (isolate 161) showed lower levels of this glucosinolate 15 days post-inoculation. NGLBR followed the same pattern for control and 161-inoculated plants. On the contrary, leaves inoculated with the avirulent isolate 130 showed a markedly decrease at 10 days post-inoculation. Leaves of controls and leaves of plants inoculated with the virulent isolate 161 showed the highest levels of this glucosinolate 15 days post-inoculation. 4-MeO-3ind glucosinolate followed the same pattern for control and 130-inoculated leaves, its levels decreased at 10 days post-inoculation, followed by an increase at 15 days post-inoculation. On the contrary, leaves infected with the virulent isolate 161 showed a markedly increase in the amount of 4-MeO-3ind glucosinolate 10 days post-inoculation as compared with leaves from controls and from non-infected plants The other indole-glucosinolate 4-OH-3ind was found in increased levels in leaves of infected plants using the virulent isolate 161, as compared with the uninoculated controls and leaves of plants inoculated with 130, uninfected leaves, at 15 days post-inoculation (almost 20 times higher amounts).

Four other glucosinolates were detected in leaves viz. GL-1, GL-2, GL-3 and GL-4. GL-1 was only found 15 days post-inoculation, in the same amounts for all treatments. GL-2 was only found in leaves of plants inoculated with the virulent isolate 161, 10 days post-inoculation and the other GL-4 was found in detectable amounts only in leaves of controls and those of plants inoculated with the avirulent isolate 130, 15 days post-inoculation (see figure-3.51-).

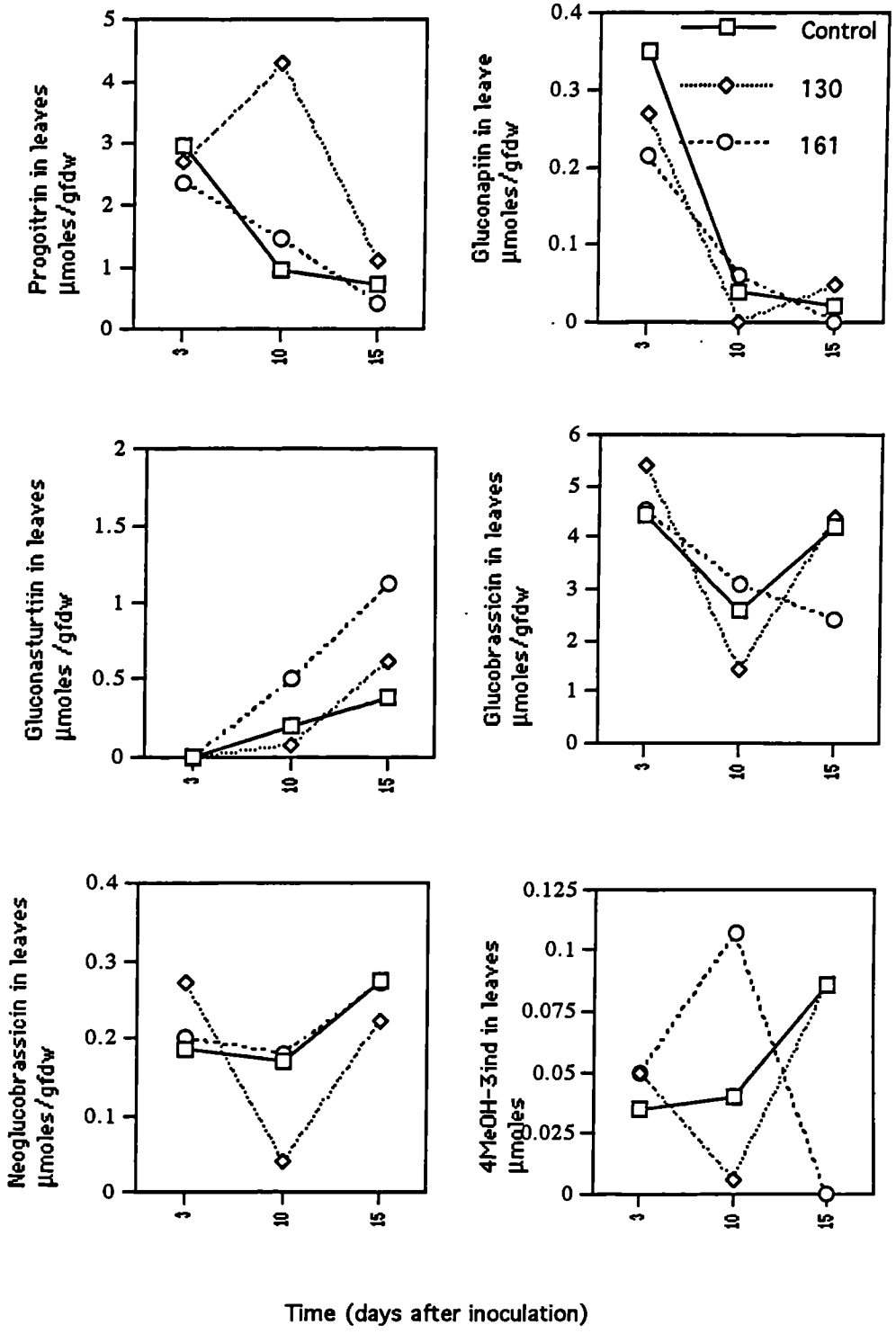


Figure-3.50- Graphic representation of the mean of duplicate determinations for PRG, GLN, GLNS, GLBR, NGLBR and 4-MeO-3ind glucosinolates in the leaves of control uninoculated plants, plants inoculated with an avirulent isolate of a non-pathogenic haploid strain of *V.dahliae* and plants inoculated with a virulent isolate 161 of a diploid pathogenic strain viz *V.d.longisporum* 3, 10 and 15 days post-inoculation (for key to glucosinolate acronyms see Table-3.39-). Plants were 2 week-old at inoculation.

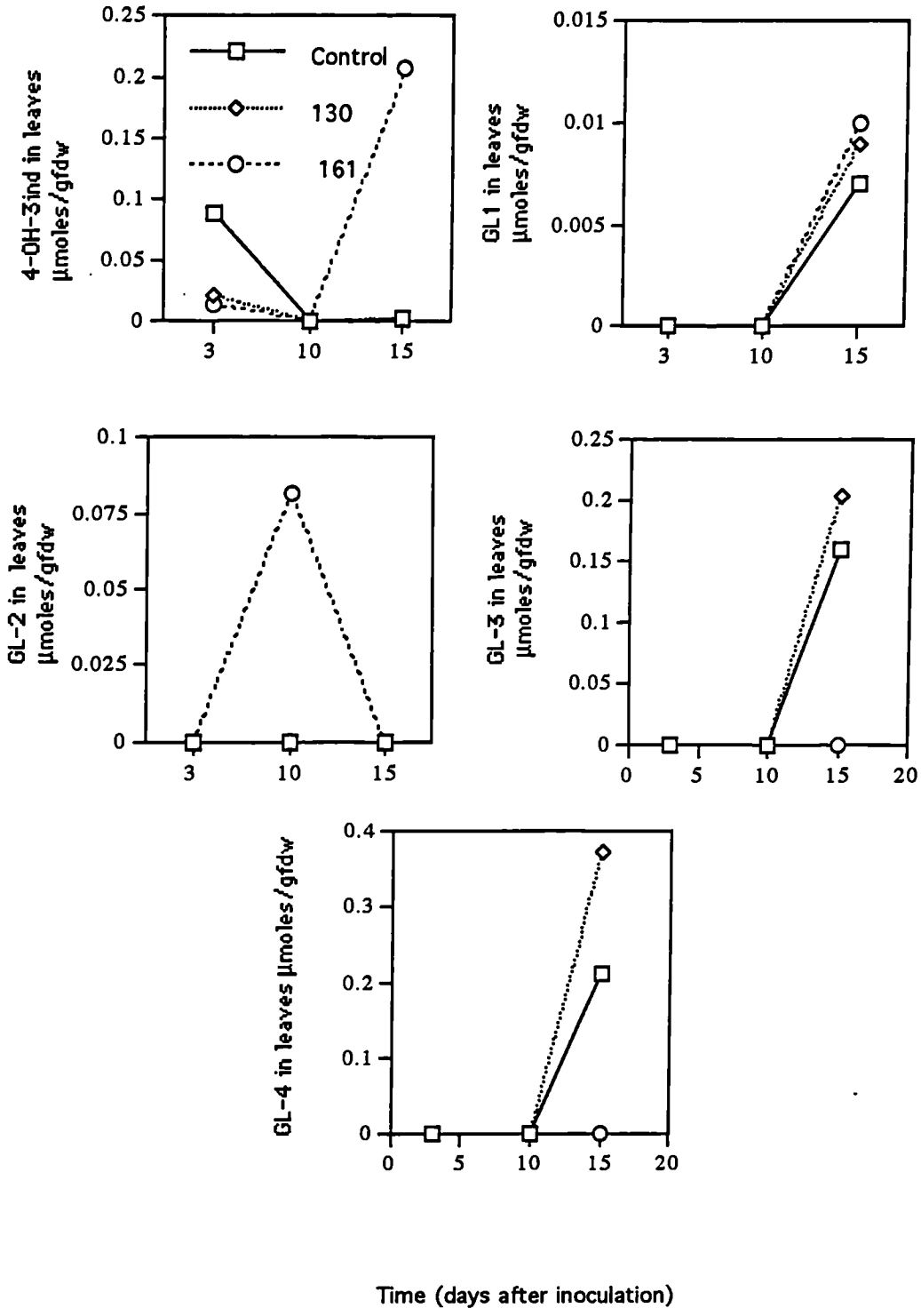


Figure-3.51 Graphic representation of the mean of duplicate determinations for 4-OH-3ind, GL-1, GL-2, GL-3 and GL-4 glucosinolates in the leaves of control uninoculated plants, plants inoculated with an avirulent isolate 130 of a non-pathogenic haploid strain of *V.dahliae* and plants inoculated with a virulent isolate 161 of the diploid pathogenic strain viz *V.d.longisporum* at 3, 10 and 15 days post-inoculation (for key to glucosinolate acronyms see Table-3.39-). Plants were 2 week-old at inoculation.

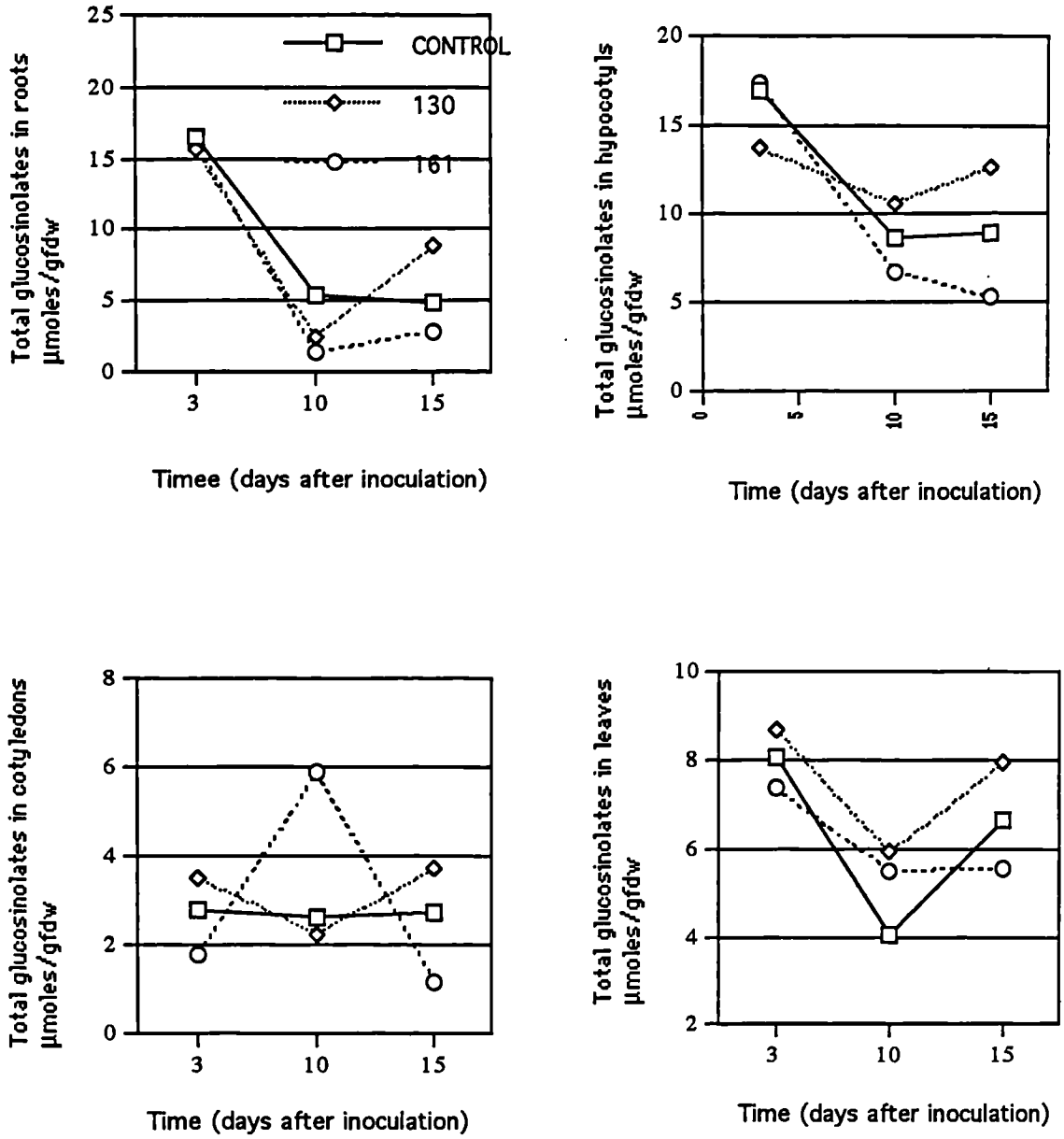


Figure-3.52- Graphic representation of the mean of duplicate determinations of total glucosinolates (summation of individual glucosinolates) in roots, hypocotyls, cotyledons and leaves of control uninoculated plants, plants inoculated with an avirulent isolate 130 of a non-pathogenic haploid strain of *V.dahliae* (tomato) and plants inoculated with the virulent isolate 161 of the diploid pathogenic strain viz. *V.d.longisporum* at 3, 10 and 15 days post-inoculation. Plants were 2 week-old at inoculation.

Table-3.41- Summary of glucosinolates, % increase* in "130-inoculated" plants at 15 days post inoculation.

GLUCOSINOLATES	ROOTS	HYPOCOTYLS	COTYLEDONS	LEAVES
PRG	88	76	160	53
GLNP	538	NP	D	135
GLNS	76	D	-	63
GLBR	310	92	48	4
NGLBR	74	73	47	D
4-OH-3ind	150	NP	NP	D
4-MeO-3ind	-	406	75	Control levels
GL-1	260	300	D	29
GL-3	NP	NP	NP	28
GL-4	NP	NP	NP	76
GL-5	NP	D	NP	NP

* As compared with non-inoculated healthy controls at 15 days post inoculation

-Undetectable amounts

D: Decreased as compared with control

NP: Not present in any treatment

Table-3.42- Summary of glucosinolates, % decrease* in "161-inoculated" plants at 15 days post inoculation.

GLUCOSINOLATES	ROOTS	HYPOCOTYLS	COTYLEDONS	LEAVES
PRG	34	Control levels	85	40
GLNP	100	NP	I	100
GLNS	85	100	I	I
GLBR	I	25	77	43
NGLBR	Control levels	36	37	Control levels
4-OH-3ind	100	NP	NP	I
4-MeO-3ind	I	I	27	100
GL-1	I	I	55	I
GL-3	NP	NP	NP	100
GL-4	NP	NP	NP	100
GL-5	NP	NP	NP	NP

* As compared with non-inoculated healthy controls at 15 days post inoculation
-Undetectable amounts
NP: Not present
I:increase

Table-3.43- Summary of glucosinolates, % increase* in both "161-, 130-inoculated" plants at 3 days post inoculation.

GLUCOSINOLATES	130R	161R	130L	161L
GLBR	29	879		
4-MeO-3ind	189	100	4.28	4
NGLBR			46	8

* As compared with non-inoculated healthy controls

Table-3.44- Summary of glucosinolates, % increase* in both "161-, 130-inoculated" plants at 10 days post inoculation.

GLUCOSINOLATES	130R	161R	130H	161H	130C	161C	130L	161L
GLBR	100	100	50	73	18	295		
4-MeO-3ind	120	100	118	165	25	158		
PRG							353	53

* As compared with non-inoculated healthy controls

Table-3.45- Summary of glucosinolates, % increase* in both "161-, 130-inoculated" plants at 15 days post inoculation.

GLUCOSINOLATES	130R	161R	130H	161H	130L	161L
GLBR	310	392				
GL-1	100	500	300	2700		
GLNS					63	195
4-MeO-3ind			406	135		

* As compared with non-inoculated healthy controls

Table-3.46- Summary of glucosinolates, % increase* only in "161-inoculated" plants at 3, 10, 15 days post inoculation.

3 Days Post- Inoculation

GLUCOSINOLATES	ROOTS	HYPOCOTYLS	COTYLEDONS	LEAVES
NGLBR	12			
PRG		14		

10 Days Post- Inoculation

GLUCOSINOLATES	ROOTS	HYPOCOTYLS	COTYLEDONS	LEAVES
PRG	17			
GLNP	100			50
4-MeO-3ind				168
GLNS				150
NGLBR				6
GLBR			295	19
GL-2				168

15 Days Post- Inoculation

GLUCOSINOLATES	ROOTS	HYPOCOTYLS	COTYLEDONS	LEAVES
GLNP			60	
GLNS			100	
GL-5		186		
4-OH-3ind				10250
4-MeO-3ind	100			

3.3.5. Compositional changes in aliphatic glucosinolates, indole glucosinolates and the aromatic glucosinolate gluconastartiin in roots, hypocotyls, cotyledons and leaves of control uninoculated plants, plants inoculated with either a virulent isolate: 161, or an avirulent isolate: 130, at 3, 10 and 15 days post-inoculation.

For each day-treatment, the values obtained were summed for indole, aliphatic and gluconastartiin (GLNS) glucosinolates separately and these were expressed them as % percentage of total glucosinolates. The transformed data are presented in Tables -3.47-, -3.48-,-3.49-, and -3.50-.

Although both indole and GLNS glucosinolates can be classified as aromatic glucosinolates, indole glucosinolates are derived from tryptophan whereas GLNS is derived from phenylalanine therefore they represent the end-products of two different pathways.

In roots, indole glucosinolates accounted for the major percentage of the total glucosinolate composition for all treatments at 3 days post-inoculation and the proportion of indole glucosinolates was higher for '161 inoculated' plants, due to the undetectable amounts of the aromatic glucosinolate GLNS. At 10 days post-inoculation GLNS were accounted for the major percentage of the total glucosinolate composition for control, uninoculated plants but indole glucosinolates accounted for the major proportion of total glucosinolates in inoculated with either the virulent (161) or the avirulent isolate 130. At 15 days post-inoculation GLNS accounted for the major proportion of total glucosinolates both for control and '130-inoculated' plants, whereas indole glucosinolates predominated in 161 inoculated plants. This was due to the increased levels of GLNS in '130-inoculated' plants, thus increasing the proportion of GLNS to that of control plants. The roots of '161-inoculated' had very low levels of this aromatic glucosinolate and indole glucosinolates remained at a higher proportion of the total glucosinolates for all these sampling times post-inoculation.

For hypocotyls aliphatic glucosinolates accounted for the higher percentage in the composition of total glucosinolates for all treatments at 3 days post-inoculation. At 10 days post-inoculation, indole glucosinolates showed the higher percentages for all treatments, and '161-inoculated' hypocotyls had an increased percentage of indole glucosinolates as compared with both the control and '130 inoculated' hypocotyls, due to the low percentage of GLNS. At 15 days post-inoculation, GLNS accounted for the highest percentage in control hypocotyls and indole glucosinolates for the '130-inoculated' and '161-inoculated' hypocotyls. One of the most noticable effects was the very high proportion of indole glucosinolates in '161-infected' hypocotyls, due largely to an apparent suppression of GLNS synthesis in these tissues. Although indole glucosinolates showed the highest percentage of the total for '130-inoculated' hypocotyls, GLNS was also relatively high.

For cotyledons, indole glucosinolates accounted for the highest percentage in the composition for all treatments at 3 days post-inoculation whilst GLNS was undetectable amounts in all treatments at this time. At 10 days, indole glucosinolates showed the highest proportion for all treatments; cotyledons of '161-inoculated' plants reflected increased proportional levels as compared with controls and '130-inoculated' plants. At 15 days post-inoculation, indole glucosinolates accounted for the highest percentage

in the composition of all treatments and '161-inoculated' tissues had the lowest percentage due to the increase in GLNS that was undetectable in tissues for both control and '130-inoculated'.

In leaves at 3 days post-inoculation, indole glucosinolates were accounted for the highest percentage in the composition of leaf glucosinolates for all treatments. At 10 days post-inoculation, again indole glucosinolates showed the largest percentage for control, and '161 inoculated', plants. The leaves of '130-inoculated' plants showed aliphatic glucosinolates to account for the highest percentage in the composition of glucosinolates in leaves. At 15 days post-inoculation, indole glucosinolates again accounted for the highest percentage in the composition of glucosinolates for all treatments. One very noticable effect was that the proportion of GLNS in leaves of '161-inoculated' plants was higher as compared with both control and '130-inoculated' plants by 10 and 15 days post-inoculation.

Table-3.47- Proportion of aliphatic glucosinolates, indole glucosinolates and gluconastartiin (% total) found in roots of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolates	Control	Inoculated '130'	Inoculated '161'
3 days p.i.			
ALIPHATIC	15~	16	13
INDOLE	60[1]	66	87
GLNS	25	18	0
TOTAL µmoles/gfdw	18.87	15.58	16.10
10 days p.i.			
ALIPHATIC	6	6	13
INDOLE	38	55	51
GLNS*	56	39	36
TOTAL µmoles/gfdw	5.44	4.61	2.98
15 days p.i.			
ALIPHATIC	4	5	5
INDOLE	28	29	78
GLNS	68	66	17
TOTAL µmoles/gfdw	4.88	8.72	2.81

~Each value represents the proportion of each glucosinolate group expressed as a percentage of total glucosinolates (% total amount of glucosinolates). **Aliphatic**: PRG; Aromatic: **Indole**: GLBR, NGLBR, 4MeO-3ind and 4-OH-3ind; and **GLNS** (*See text for explanation).

[1]The number in bold represents the higher percentage.

Table-3.48- Proportion of aliphatic glucosinolates, indole glucosinolates, and gluconastartiin (% total) found in hypocotyls of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolates	Control	Inoculated '130'	Inoculated '161'
3 days p.i.			
ALIPHATIC	55~	58[1]	59
INDOLE	45	42	41
GLNS*	0	0	0
TOTAL µmoles/gfdw	16.88	13.68	17.34
10 days p.i.			
ALIPHATIC	37	32	30
INDOLE	38	45	57
GLNS	25	23	13
TOTAL µmoles/gfdw	8.57	9.92	6.53
15 days p.i.			
ALIPHATIC	10	11	21
INDOLE	41	55	79
GLNS	49	34	0
TOTAL µmoles/gfdw	8.27	12.13	3.32

~Each value represents the proportion of each glucosinolate group expressed as a percentage of total glucosinolates (% total amount of glucosinolates). **Aliphatic**: PRG, GLNP, NPL; Aromatic: **Indole**: GLBR, NGLBR, 4MeO-3ind and **GLNS** (*See text for explanation).

[1]The number in bold represents the higher percentage.

Table-3.49- Proportion of aliphatic glucosinolates, indole glucosinolates, and gluconasturtiin (% total) found in cotyledons of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolates	Control	Inoculated '130'	Inoculated '161'
3 days p.i.			
ALIPHATIC	22~	32	23
INDOLE	**78**[1]	**68**	**77**
GLNS*	0	0	0
TOTAL µmoles/gfdw	2.72	3.41	1.79
10 days p.i.			
ALIPHATIC	29	27	12
INDOLE	**58**	**69**	**85**
GLNS	13	4	3
TOTAL µmoles/gfdw	2.6	2.24	5.83
15 days p.i.			
ALIPHATIC	8	7	15
INDOLE	**92**	**93**	**52**
GLNS	0	0	33
TOTAL µmoles/gfdw	2.6	3.85	1.34

~Each value represents the proportion of each glucosinolate group expressed as a percentage of total glucosinolates (% total amount of glucosinolates). **Aliphatic**:PRG, GLNP; Aromatic: **Indole**: GLBR, NGLBR, 4MeO-3ind, 4-OH-3ind; and **GLNS** (*See text for explanation).

[1]The number in bold represents the higher percentage.

Table-3.50- Proportion of aliphatic glucosinolates, indole glucosinolates, and gluconasturtiin (% total) found in leaves of inoculated and uninoculated OSR plants (cv. Cobra).

Glucosinolates	Control	Inoculated '130'	Inoculated '161'
3 days p.i.			
ALIPHATIC	41~	34	35
INDOLE	**59**[1]	**66**	**65**
GLNS	0	0	0
TOTAL µmoles/gfdw	8.03	8.71	7.26
10 days p.i.			
ALIPHATIC	25	**73**	28
INDOLE	**70**	25	**63**
GLNS	5	2	9
TOTAL µmoles/gfdw	4	5.85	5.39
15 days p.i.			
ALIPHATIC	13	18	10
INDOLE	**80**	**72**	**65**
GLNS	7	10	25
TOTAL µmoles/gfdw	5.7	6.46	4.45

~Each value represents the proportion of each glucosinolate group expressed as a percentage of total glucosinolates (% total amount of glucosinolates). **Aliphatic**: PRG, GLNP; Aromatic: **Indole**: GLBR, NGLBR, 4MeO-3ind, 4-OH-3ind; and **GLNS** (*See text for explanation).

[1]The number in bold represents the higher percentage.

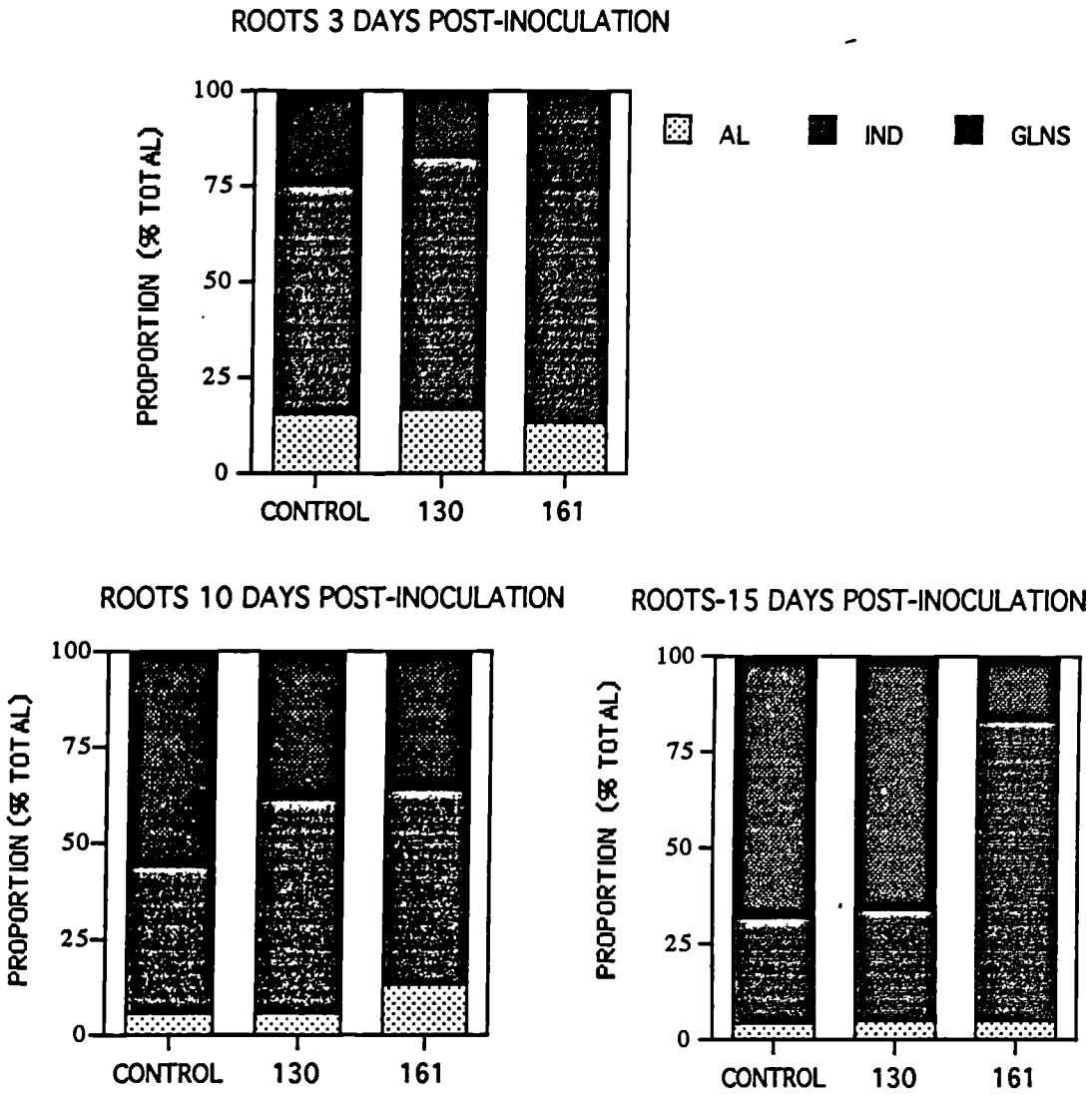
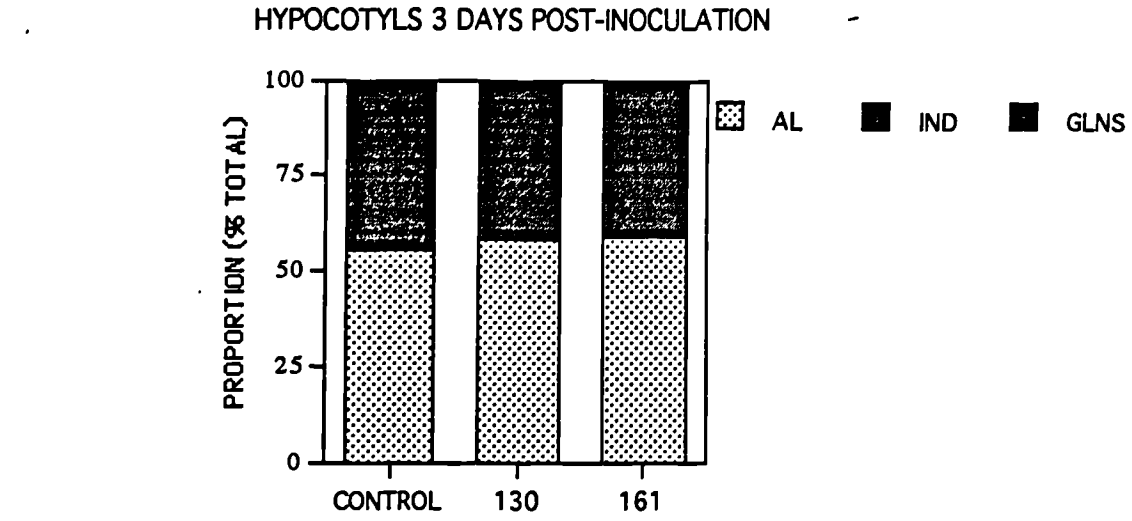
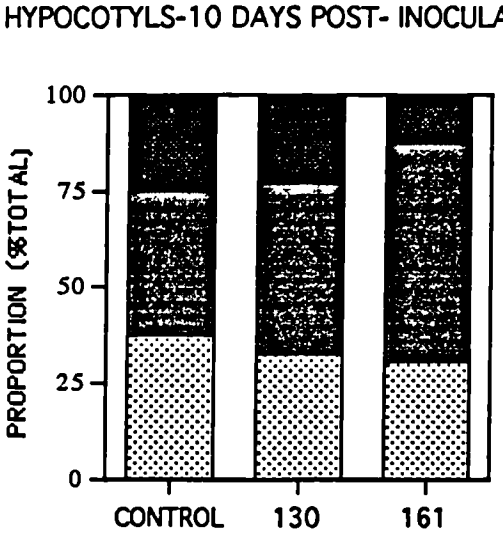


Figure-3.53- Proportion (% total) of aliphatic (AL), indole (IND) and gluconasturtiin (GLN) in roots of control uninoculated plants, '130-inoculated' plants and '161-inoculated' plants. For details see Table-3.47-.





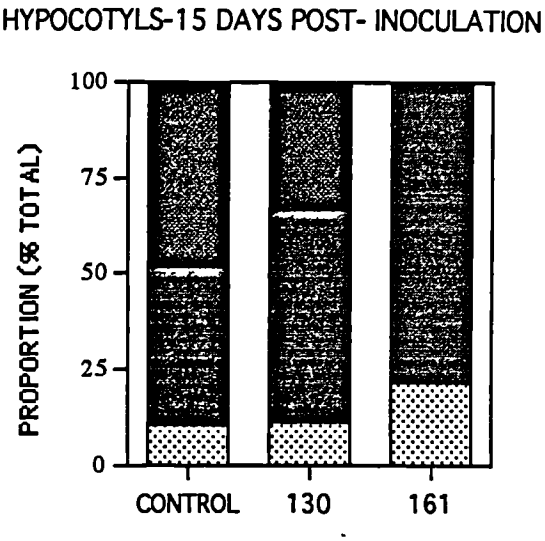


Figure-3.54- Proportion (% total) of aliphatic (AL), indole (IND) and gluconasturtiin (GLN) in hypocortyls of control uninoculated plants, '130-inoculated' plants and '161-inoculated' plants. For details see Table-3.48-.

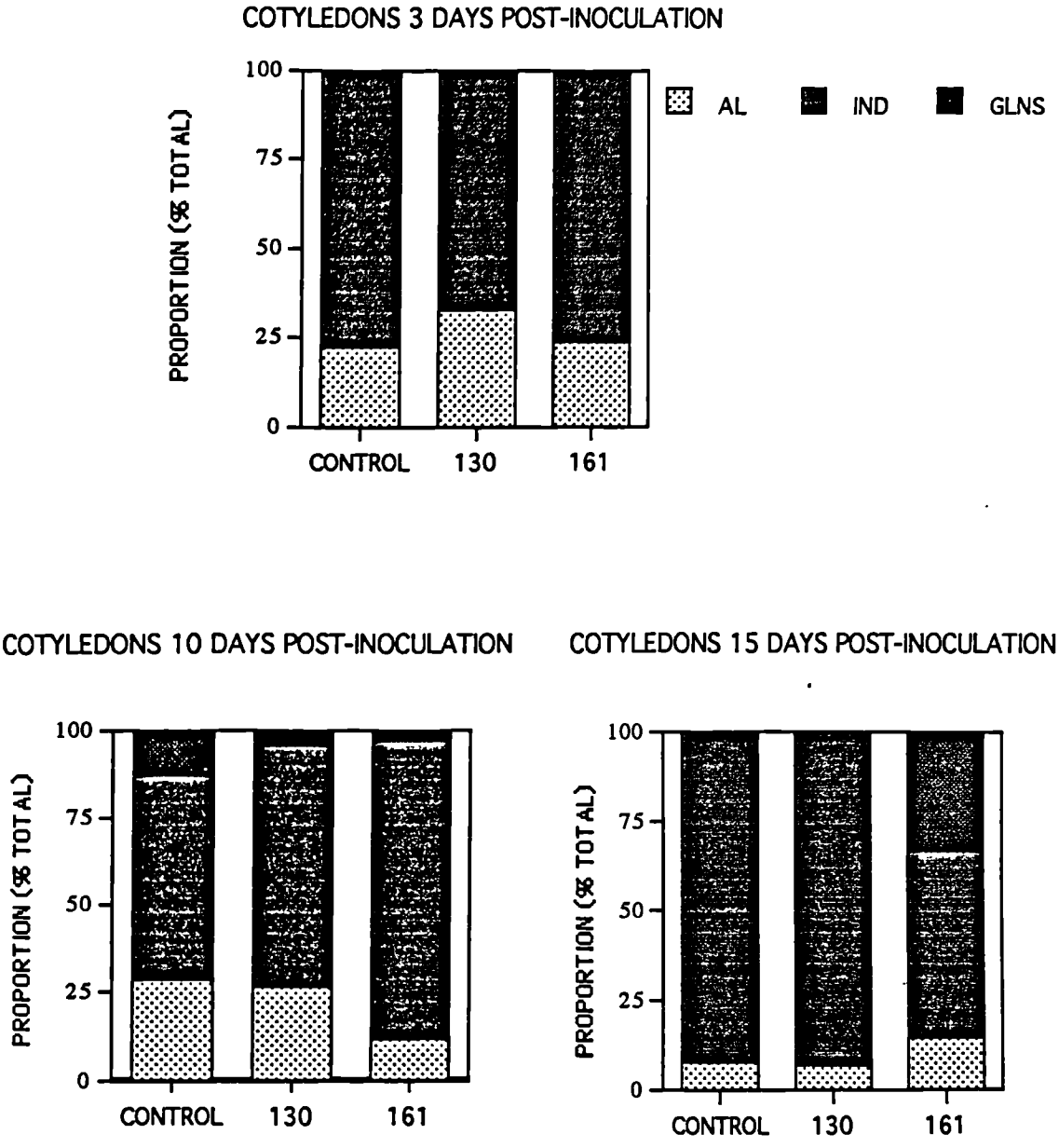


Figure-3.55- Proportion (% total) of aliphatic (AL), indole (IND) and gluconasturtiin (GLN) in cotyledons of control uninoculated plants, '130-inoculated' plants and '161-inoculated' plants. For details see Table-3.49-.

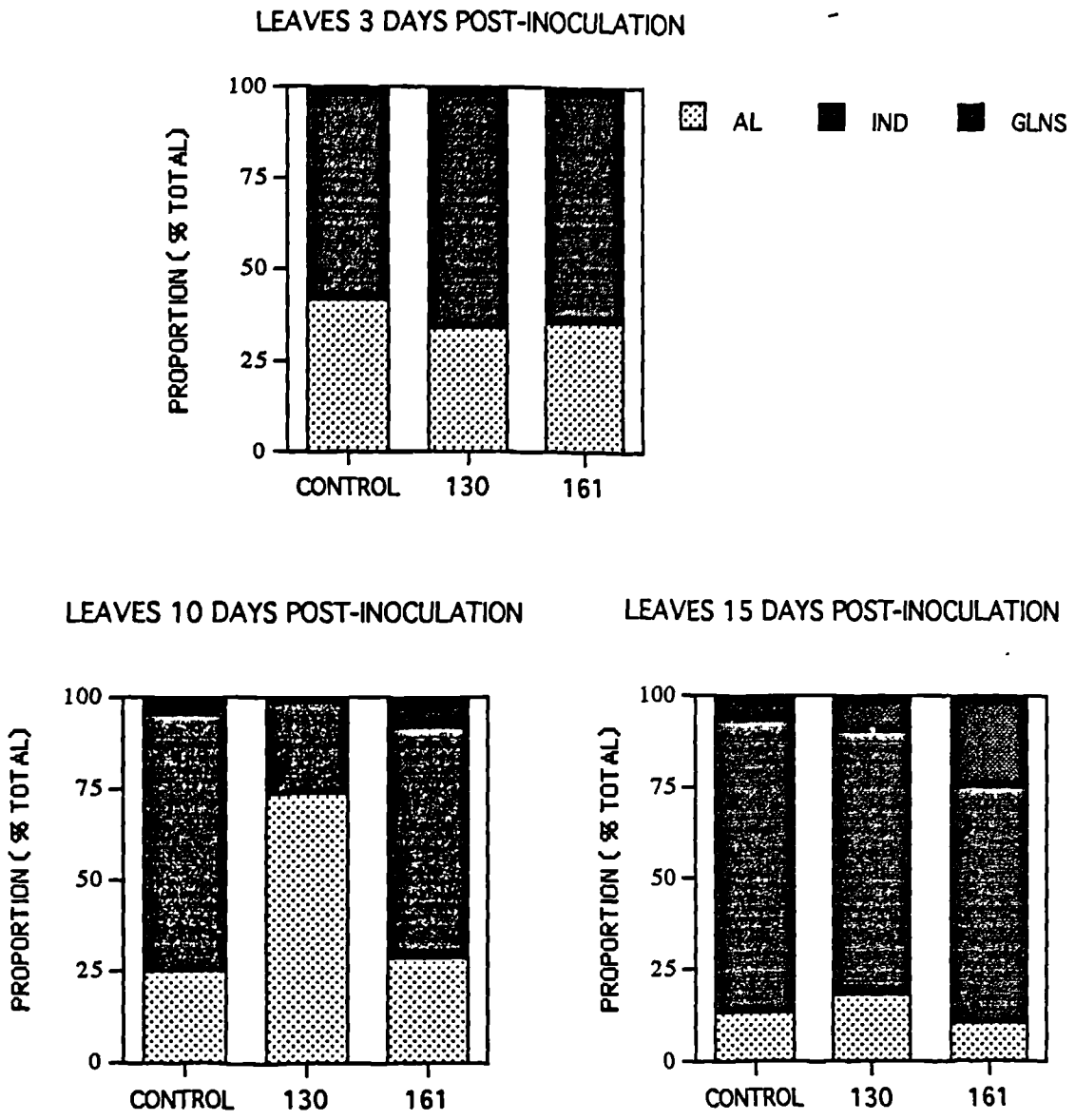


Figure-3.56- Proportion (% total) of aliphatic (AL), indole (IND) and gluconasturtiin (GLN) in leaves of control uninoculated plants, '130-inoculated' plants and '161-inoculated' plants. For details see Table-3.50-.

4. Discussion

Characterization of the pathogen

Observations made, for the first time in most cases, in the present study of conidia of many different *V.dahliae* isolates obtained from different Brassica diseased hosts from different countries (i.e. *V.d.longisporum* Stark), showed a marked difference in conidial length compared with that of typical *V.dahliae* strains from other hosts. When ungerminated spores from Brassica isolates were harvested from PDA semi-synchronous cultures, to standardise the age, and measured unstained with the aid of haemocytometer, they were found to have almost double the conidial length of the typical *V.dahliae*, thus resembling the 3 known diploids identified by Jackson & Heale (1985) as *V.d.longisporum* Stark, 1961. The 26 isolates from Brassica hosts and related species and the 3 'well-known' naturally occurring diploids described by Jackson & Heale (ibid.) (isolates 195, 161, 162), formed a separate group (viz. Group B) from the other 10 isolates from other hosts (viz. Group A). Group B consisted of 29 isolates which possessed long conidia (of 7μm or more), while group A consisted of isolates which possessed short conidia (of 5.5μm or less), according to mean spore lengths of 50 randomly chosen spores. In Group B, all isolates, except one (161, sugarbeet), had been isolated from Brassicaceae hosts. It is possible that this isolate (161) came from infected sugar beet grown in soil previously intensively cropped with rape in Sweden, but this has not been confirmed. In Group A, four isolates were from Cruciferous-hosts: one isolate from Brussels sprouts, (111, UK), one isolate from the weed, Shepherd's purse (362, USA), one isolate from stock, (G17, Germany) and one isolate from oilseed rape (G22, Germany). When the isolate G22 was first obtained from Germany in 1991, spore length measurements indicated that it produced long spores, as did the other oilseed rape isolates (mean length 8.70±0.23μm); however, after one year of sub-culturing it started to produce the short-length typical spores of *V.dahliae* with mean length almost half (4.607±0.061μm) of the parental 'long-spored' culture of G22. These spore length measurements and the apparent 'breakdown' of the 'long-spored' isolate G22 to a typical haploid, short-spored recombinant were the first indication of its original diploidy, as it is often found (see below) that there is a good correlation between cell size, nuclear volume and DNA content and ploidy in various organisms including animals, plants and fungi.

There are several examples among plant-pathogenic fungi where diploid spores are twice as large as haploid spores, *Ustilago violaceae*, *Fusarium oxysporum* Schl. f. *pisi*, *Ascochyta imperfecta*, *V.dahliae*, *V.albo-atrum*, *V.tricorpus*, (Tinline & MacNeil, 1969; Tolmsoff, 1972; Day, 1972). Clutterbuck (1969), who studied cell volume in haploid and diploid conidiophores of *Aspergillus nidulans*, showed that the volume was proportional to ploidy and DNA content. Spore length measurements provided a reliable distinction between haploid and diploid segregants in some Imperfect fungi known to have a parasexual life cycle(Pontecorvo, 1956). Hastie (1964), who first demonstrated the parasexual cycle in *Verticillium*, obtained heterozygous nuclei from heterokaryons (produced from auxotrophic complementary mutants) which yielded two classes of novel genotypes: (i) colonies with large uninucleate conidia (11μm) which were segregating for all markers introduced from the parents and were unstable (diploids), and (ii) colonies with small conidia (6μm) which were very stable (haploids). Among *Verticillium* species, the known

naturally occurring diploids viz. 195, 161, 162, (Stark, 1961; Typas & Heale, 1977; Puhalla & Hummel, 1984; Jackson & Heale, 1985), have double the conidial length of the typical haploid *V.dahliae*, and have been named *V.d.longisporum* Stark (Jackson & Heale,1985). Additionally, conidial volume and length have been used by previous authors (Stark, 1961; Hastie, 1964; Puhalla & Mayfield, 1974; Typas & Heale, 1976, 1977, 1978; Jackson & Heale, 1985; Horiutchi *et al.*, 1990) as one criterion of diploidy in naturally occurring diploids in species of *Verticillium*, as well as for diploids that had been synthesized in the laboratory through the parasexual cycle; these authors reliably established the level of diploidy, especially when it was also verified by cytological tests (nuclear staining and genetic analysis) e.g. Typas & Heale (1980), and when environmental conditions and culture age were standardized (Pelletier & Aube, 1970; Jackson & Heale, 1985). Thus, spore length among fungi producing **mononucleate**, elliptical, or elongated spores is, in general, a valuable indicator of ploidy and the detection of large conidia above the haploid size range can indicate naturally occurring diploids (Hastie & Heale, 1984). However, as pointed by Tolmsoff (1983) additional information is required to establish and confirm the types of ploidy involved.

To further investigate the ploidy levels of these *Brassica* isolates in the present study, the nuclear diameters of DAPI-stained nuclei were measured. All isolates of *V.dahliae* and *V.d.longisporum* tested had uninucleate conidia, which is in agreement with observations by previous authors (Hastie, 1962,1964; Heale *et al.*, 1968; Puhalla & Mayfield, 1974; Typas & Heale, 1980; Jackson & Heale, 1985). According to their nuclear diameter, isolates were clustered into two main groups. Isolates of Group A (with mean spore length of 5.5μm or less), had nuclear diameters with a mean range of 0.92μm-1.03μm, while isolates of Group B (with mean spore length of 7μm or more), had nuclear diameters with a mean range of 1.7μm-2.1μm, although there were 5 isolates that showed intermediate measurements (three isolates in Group A: 140, 362 and G1, and two isolates in Group B: 84120 and F-617 with nuclear mean diameters ranging from 1.4-1.6μm). The 3 intermediate isolates in group A, although showing a relatively larger fluorescent area as compared with the other isolates in the same group, exhibited fluorescence which was less bright than the fluorescence of the other 2 isolates with intermediate spore length in Group B which emitted a bright blue colour; thus, it cannot be concluded from this observation that these 5 isolates had the same amount of DNA. All the measurements on other isolates were made on brightly fluorescent, spherical nuclei, and as DAPI specifically binds to DNA, the fluorescent area can be related to the DNA content. It was noticed that there was a very small number of spores (almost 1%) in each sample tested that had a crescent- shaped fluorescent nucleus instead of one dense fluorescent, spherical nucleus. These nuclei, are assumed to be nuclei that had passed the G1 phase and started to divide, and for this reason these measurements were not included in this study. Heale *et al.* (1968) studied the mitotic division of nuclei, in conidia and hyphae of *V.albo-atrum*, using different nuclear stains and described 4 phases in the nuclear division; horse-shoe phase, ring phase, double ring phase and separation phase. The two first phases, as illustrated in the same paper, were similar to the ones described in the present investigation as crescent-shape nuclei, but no more observations on these unsynchronous germinated conidia were made and as only a small percentage of these nuclei were observed, it gave a good indication that the majority of conidia used for this study were in G1 phase. Acridine-stained nuclei were more laborious to obtain and quench easily and for this reason only DAPI was used for measurements of nuclear diameters.

Following further these indications of diploidy, DNA content was estimated by Feulgen DNA microdensitometry, and compared to an internal control (isolate 161 from sugarbeet that had been previously reported to be diploid by Puhalla & Mayfield, 1974; Jackson & Heale, 1985). Isolates of the Group B (except one isolate G19, isolated from rape) had c. 1.75X the amount of DNA of the isolates of Group A, a value that indicates diploidy or partial diploidy. The range of values (in arbitrary units) for the haploid isolates was 0.63-0.95 and the mean value 0.8, while isolates of the Group B had a range of 1.26-1.73 and mean value 1.4. The full diploid range would be 1.26-1.9 with a mean value of 1.6, indicating that there is c. **12.5% less DNA** than it would be if it was a complete homozygous diploid, or a hybrid between *V.dahliae* and *V.albo-atrum*. The original Stark isolate (195) was found by Tolmsoff (1983) to contain 0.076 pg of DNA / nucleus ; Typas & Heale (1980) found 0.025-0.030 pg of DNA /nucleus for an haploid isolate of *V.dahliae* from pepper, and c. double that amount of DNA /nucleus for a diploid strain of *V.d.longisporum* i.e. 0.050-0.060 pg of DNA/nucleus, which agrees with Tolmsoff results even though different techniques were used. Actual measurements of extracted DNA were not performed in this present study, but because the same technique as that employed by Typas & Heale (1980) was used, isolates of group B were estimated to contain **0.043-0.052 pg of DNA / nucleus**. One isolate viz. G19 from group B gave DNA values of 0.8 corresponding to the haploid level, as compared with the 1.47 for the internal (control) diploid isolate 161; this was the first indication that this isolate was not diploid, because the value previously obtained for its mean spore length was 9.100±0.014μm, i.e. in the range of the other diploid isolates in Group B; also the nuclear diameter measurements (1.742±0.044μm) on DAPI stained nuclei did not reveal the same result. G19 was the only isolate which produced spores which had a mean length that did not correlate with the amount of DNA /nucleus and also the only one isolate where the results from DAPI-staining and Feulgen staining did not correlate. The isolate G22 gave DNA values of 0.85, agreeing with its haploid spore length as expected. Some measurements were also performed for the isolate 111 (Brussels sprouts, UK) and this isolate also had a higher amount of DNA compared with the other haploid isolates, giving the first indication of diploidy or partial diploidy for this isolate.

Additional evidence to support the 'diploid' state of these Crucifer-strains comes also from the fact that previous authors could not obtain auxotrophic mutants by UV irradiation, compared with the high frequency of auxotrophs obtained from haploid conidia of *V.dahliae, V.tricorpus* or *V.albo-atrum* (Fordyce & Green, 1964; Hastie 1973, 1978; Puhalla 1973; Puhalla & Mayfield, 1974; Typas & Heale, 1977,1978; Clarkson & Heale 1985a, b, c). Ingram (1968) who studied the original diploid isolate of *V.d.longisporum* Stark, reported that auxotrophs could not be obtained from this large-spored form but were produced from the small-spored, haploid types produced by haploidization with p-fluorophenylalanine. Similarly, Puhalla & Hummel (1983) studied the two isolates (161, 162) from Sweden and could not obtain microsclerotia colour mutants from them after UV treatment. Dr. L. Portenko (personal communication) also could not obtain auxotrophic mutants of long-spored isolates of *V.dahliae* from infected oilseed rape plants in Russia and The Ukraine. Additionally, Japanese groups working with these Cruciferous isolates could not obtain nit-mutants (Nagao *et al.*, 1994a) or melanin-deficient mutants (Nagao *et al.*, 1994b) from them in order to classify them in vegetative compatibility groups.

Attempts to obtain haploid segregants from the diploid isolates were made in the present work, using complete medium amended with chloral hydrate, an haploidization agent (Mackintosh & Pritchard,

1963), but this was not successful. Horiuchi (personal communication) tried to haploidize Japanese isolates, including the 161 isolate from sugarbeet, in p-fluorophenylalanine (p-fa) medium, but also was not successful. The same haploidization medium (p-fa) has also proved ineffective in yeasts and Basidiomycetes such as *Ustilago maydis* and *Coprinus lagopus* (Burnett, 1975). Jackson & Heale (1985) using p-fa reported the production of small-spored isolates, (thought to be haploid segregants by DNA Feulgen microdensitometry), from isolates of *V.d.longisporum* Stark (viz. 161, 162, 195). Later, Baig (1991) also produced very small-spored segregants from the same 3 diploid isolates (195,161,162) and one Japanese isolate (86207) with the same haploidization agent, but these were not very stable and were difficult to maintain in culture. Actual DNA measurements were not conducted, but they were presumed to be haploid segregants by their reduced spore size which was less than a half of that of their 'parents'. These putative haploids were found to be non-pathogenic to rape because they did not produce symptoms and the organism was never isolated from the inoculated plants.

Our difficulties in obtaining stable haploid segregants by haploidization could be due to the complex nature of these diploid isolates. Thus, it may be suggested that this stable diploid pathogen is not an homozygous diploid formed by mitotic non-disjunction of the whole chromosome sets within a single strain of *V.dahliae*; rather it can be postulated that it is more likely to be the **result of recombination in an heterozygous nucleus** formed by a fusion of two different haploid nuclei, that took place in an heterokaryon formed between two unknown parental strains, i.e. a **hybrid** of two compatible, but not complete haploid genomes (deficient for normal growth) i.e. different *V.dahliae* strains, or a *V.dahliae* and *V.albo-atrum* that complemented each other in the 'diploid' form. Additional evidence to support the hypothesis that these diploid isolates were not derived from two complete haploid genomes was also derived from the preliminary UV sensitivity experiments performed in the present work. Haploid isolates when exposed to UV show a steady decline in their survival curve because increase in dosage leads to increased mutation including lethal changes in genes. On the contrary, diploids have the complementary wild type allele to the mutated gene and thus are quantitatively less sensitive to UV exposure. Puhalla & Mayfield (1974) obtained putative diploids from complementary auxotrophs obtained by UV from the defoliating T9 isolate of *V.dahliae* from cotton and showed that these diploids were 1.7 times more resistant to killing by ultraviolet light than were haploids. The resistance to UV can be illustrated by the 'shoulder' of such survival curves. The present results from UV sensitivity experiments however revealed the opposite of that expected for true diploids; diploid isolate 161 was more sensitive to UV-exposure compared to haploid isolates 130 and G1; similarly isolate G334 was more sensitive than haploid isolate G1. These results are indicative of the complex nature of the genome of these apparently 'diploid' isolates.

Combining together all the above results, three groups were distinguished viz.: (1) isolates from different hosts that had typical small haploid spores (Group A); (2) isolates from Cruciferous hosts that had longer spores than those of the typical haploid type and appeared to be diploid by DNA Feulgen microdensitometry (Group B); (3) three isolates were exceptional with intermediate features. Thus, isolate 111 was isolated from Cruciferous host i.e. Brussels sprouts and had typical short spores, but its DNA values according to Feulgen DNA microdensitometry indicated that it was a diploid or a partial diploid. A second peculiar isolate viz. G19 which had been isolated from an infected oilseed rape plant in the field in

Germany, had long spores as did all the other rape isolates, but gave DNA values similar to that of the typical haploid isolates. The other isolate (G22) when first isolated had long spores, but after one year sub-culturing produced small spores and was shown to have typical haploid DNA values when Feulgen DNA measurements were performed.

Following these observations, one more morphological character, the formation of microsclerotia on four different media i.e. PDA, Hall & Ly medium (Hall & Ly, 1972), prune lactose yeast medium (Talboys, 1960) and rape medium (Peterka & Schlosser, 1989) was investigated to see if there were any differences between the isolates of the two putative groups (A and B) and especially for the above 3 peculiar intermediate isolates (111,G19, G22).

Isolates of both A and B groups formed only microsclerotia, type M, according to the description of Isaac (1949). Hall & Ly (1972) studied the formation of microsclerotia in *V.dahliae* viewed with the light microscope and described 3 stages: a) Initiation, where areas on a hypha swell and develop numerous septa, b) enlargement, lateral budding leading to a three dimensional structure and c)maturation when the dark pigment (allomelanin) was developed inside the walls of the structures formed. The same stages were identified in the present study on PDA medium of 10-day-old cultures and differences between the two groups (A and B) of isolates were observed in all developmental stages, but especially in the last two. All isolates started to produce microsclerotia on PDA medium by the 4th day after inoculation. Isolates of the Group B, as compared with isolates of Group A, produced more microsclerotia and conidia, but this could be due to the shorter time that these isolates were maintained on culture media (Group B isolates were isolated during the last 8 years, whereas most isolates in Group A had been kept in culture for periods longer than 8 years). Isolates in both groups formed microsclerotia that were initiated from individual cells of hyphae that became more regularly septated and subsequently grew to form chains of globose cells. At this stage (initiation), hyphae of isolates of Group A were less frequently septate and subsequently formed globose cells, as compared with those of isolates of Group B. Brown & Wyllie (1970) described the development of microsclerotia of *V.albo-atrum* (sensu stricto=*V.dahliae*) ultrastructurally by transmission and scanning electron microscopy and described these chains of globose cells in the beginning of formation of microsclerotia and they also described anastomosis. Anastomosis between hyphae and globose cells were also observed in this present study, and was more frequent for isolates of Group B. Typical haploid isolates (Group A) formed a three dimensional structure, (type M2) (budding at all planes at stage b), compared with the more elongate two dimensional structure (M1) (budding more at two planes) exhibited by the diploids (Group B). Actually, the original description of Reinke and Berthold (1879) for *V.albo-atrum* describes what can also be interpreted as the initiation of microsclerotia formation of *V.dahliae*. The mature microsclerotia (3 months old) formed on PDA of the isolates of the two groups A and B were also observed to be different in the present study. M1 microsclerotia formed by Group B were connected with melanised globose cells that had not undergone budding, thus giving an elongate appearance to the final structure. On the contrary, M2 microsclerotia formed by Group A were compact and more individual. Thus, the formation of microsclerotia on PDA, also differentiated the isolates into the two main groups. In summary isolates of Group A, (haploids) formed typical, compact M2 microsclerotia whereas isolates of Group B, (diploids) formed elongate M1 microsclerotia. The only definite exception was the isolate G19

which produced peculiar microsclerotia that could not be definitely classified in one or the other type, not being elongate or compact enough to be classified as M1 or M2. In addition, isolate 111 and 195 did not easily produce microsclerotia on PDA and G22 produced typical M2 microsclerotia as expected for a haploid Group A type isolate.

Hall & Ly medium, diluted by a factor of 30, was used by Horiuchi *et al.*, (1990), and reliably distinguished the groups pathogenic to Solanaceous plants (*V.dahliae* isolates obtained from different hosts: Groups A,B,C) from those which were not pathogenic to Solanaceous plants but pathogenic to Cruciferous Group D) based upon the morphology of microsclerotia. The same dilution factor was attempted in the present study, but only some isolates of group B produced microsclerotia on this diluted medium and for this reason the same medium undiluted with 2mg/ml KNO_3 and 0.6 mg/ml glucose was adopted since Hall & Ly (1972) had shown that under these low levels of glucose, growth was restricted and microsclerotia were produced at the expense of hyaline hyphae. Again in this medium isolates of Group B produced microsclerotia more abundantly and were of type M1 while only 4 isolates of Group A produced microsclerotia of type M2. G19, 111 and 195 (original *V.d.longisporum*, Stark, 1961) did not produce microsclerotia on this medium. Horiuchi *et al.* (1990) also found difficulty in obtaining microsclerotia in this relatively 'old' isolate 195. G22 when first obtained, produced elongate microsclerotia on this medium but after it 'broke down' to the haploid state, did not produce any microsclerotia on this specific medium.

Prune Yeast Agar medium was proposed by Talboys (1960) to enable a ready microscopic identification and distinction between *V.albo-atrum* and *V.dahliae*. All isolates grew well and produced microsclerotia as well as in PDA medium. All typical isolates of Group A produced type M2 microsclerotia and typical isolates of Group B produced type M1 microsclerotia. Isolate G19 produced both types of microsclerotia, (type M1 embedded in the medium and type M2 on the surface of the medium), isolate 111 produced type M1 microsclerotia and isolate 195 failed to produce microsclerotia. On this medium also, differences were identified among the 3 Japanese isolates of Group B: isolate 86207 produced thinner and longer, irregular microsclerotia than the other two (84120, 84013) isolates.

In rape medium, only 4 isolates of the Group A produced microsclerotia and these were of typical spherical compact microsclerotia (M2 type), the other isolates in the some group failed to grow. On the contrary, all typical isolates of Group B (except isolate FI) produced microsclerotia on this medium and were elongate (M1). The isolate G19 produced the two types of microsclerotia (M1 type and M2 type) on this medium. Isolates 111 and 195 failed to grow under these conditions.

Thus, the two groups (A and B) could more or less be clearly differentiated using their microsclerotial characteristics. Using the 4 different media the isolates of Group A formed typical spherical microsclerotia type (M2) whereas isolates of the Group B formed irregular, elongate microsclerotia type M1. The detection of dark pigmented cells with no lateral budding in Group B isolates gave a first indication of the possible origin of these Brassica diploid isolates as a hybrid between *V.dahliae* and *V.albo-atrum* i.e. it can be suggested that they represent a diploid hybrid between the two haploid species.. There were four exceptional isolates (viz. G22, 111, 195, and G19) that either failed to produce microsclerotia, or did not produce the typical microsclerotia of their Group classified according to their conidial length and for this reason they were investigated further.

Isolates of the two groups (A and B) also showed differences during conidiophore formation. Isolates of Group B (diploids) had longer conidiophores with 3-4 phialides/ node, while isolates of the Group A (haploids) had shorter conidiophores with 4-5 phialides (5 phialides were especially observed at the apex of the conidiophore). Thus, fewer and longer phialides/node were observed in the diploid isolates of **Group B** compared with the haploid isolates of Group A. Conidiophores were produced mainly from the globose cells that initiated microsclerotia formation in both groups, and partially hyaline variants that lacked these "initiation" globose cells formed very few conidiophores. Diploid isolates produced black microsclerotia more readily and conidiated more abundantly than haploids. Horiuchi *et al.* (1990) who studied the formation of conidiophores in their Group D (putative diploids) reported that each conidiophore possessed fewer phialide-bearing nodes than in other groups. Further, Dr. L. Portenko (personal communication) studied the conidiophores of isolates from oilseed rape that possessed long spores (i.e. diploids) and observed that their conidiophores were different from typical haploid isolates of *V.dahliae*, being irregular in length and comparable with the conidiophores observed in the artificially synthesized diploids from complementary auxotrophic haploids. This observation was another indication of diploidy because one may expect longer and fewer phialides in diploid isolates to support/accommodate the larger amount of DNA per nucleus. Hastie (1967) studied mitotic recombination in conidiophores of *V.albo-atrum* and reported one-two nuclei in each phialide, and that the nucleus of the newly formed conidium at the apex of the phialide originated from the division of the already existing nucleus in the phialide that migrated into the developing conidium, while the other nucleus remained in the phialide to divide later. Nuclei staining in conidiophores of these diploid isolates was not performed in the present study, but if we take into consideration Hastie's (ibid.) results from phialide analysis, we expect that each phialide is a cell with one or two nuclei; thus in the diploids the content of DNA in each phialide is more than it would be if they were haploids, and thus it appears that larger/ longer phialides are required to accomodate the larger amount of DNA per nucleus in the diploid.

Howell (1970), employed a medium containing tannic acid for the qualitative analysis of extracellular polyphenol oxidase (p.p.o) activity in haploid forms of microsclerotial strains of *V.albo atrum* (i.e. actually sensu stricto=*V.dahliae* Kleb.) and homozygous diploid forms derived from corresponding haploids. He reported that the haploid isolates which were pathogenic to cotton plants exhibited dark zones, whereas the diploids which were derived from highly virulent haploids were non-pathogenic (no discernible symptoms in cotton although they were present in the upper parts of the plant), failed to grow on the medium and showed no dark zones indicating low activity of extracellular p.p.o activity. Thus, a change in ploidy without any gene change, had caused suppression of p.p.o activity. Horiuchi *et al.* (1990) using the same medium, reported low activity levels of extracellular p.p.o activity for the 3 established diploid isolates of *V.d.longisporum* (195, 161, 162) and also for their 'Cruciferous ' isolates, i.e. those which did not cause disease to Solanaceous plants. Polyphenol oxidases are key enzymes in the oxidation of phenolic compounds to quinones, which are very toxic to fungi as compared with the non-oxidised forms. This visual test for p.p.o also clearly differentiated the two major groups in the present study. All haploid isolates of Group A (with the exception G22) exhibited extracellular p.p.o whereas all diploid isolates of Group B did not exhibit detectable activity. Isolate G22 was the only exception to the rule,

that although being haploid by previously established criteria, it did not exhibit extracellular p.p.o. This suggested that this isolate may be a haploid recombinant derived from an initially diploid, parental isolate. Diploid isolates 111 and 195 as expected did not exhibit p.p.o, whereas G19 behaved as an haploid and showed p.p.o activity. The two *V.albo-atrum* R & B isolates tested (which were haploid), one from lucerne and one from chrysanthemum, exhibited extracellular p.p.o. activity. All isolates under test grew well on this specific medium and produced microsclerotia by the 15th day of inoculation, first appearing in the periphery of the colony. The modified Eckert medium (1962) used by Howell (1970) contained two carbon sources: glucose (2%) and sucrose (0.5%). When the latter medium was used leaving out glucose as carbon source, the diploid isolates could not be differentiated from the haploid isolates in the present study. Only 3 isolates of Group A (viz. G1, G17, 362) and one of Group B (isolate G19) exhibited detectable extracellular p.p.o. in the absence of glucose. All the other haploids did not exhibit detectable extracellular p.p.o. This is a very interesting observation indicating the importance of the presence of glucose for the expression of extracellular p.p.o. enzyme activity in haploid *V.dahliae* cultures. Carder *et al.*, (1987) studied the *in vitro* production of extracellular polygalacturonase, pectin lyase and cellulase from hop isolates of *V.albo-atrum* in relation to their virulence to hop, on 3 different media containing glucose, pectin or acetone-extracted hop tissue as carbon source; they found that virulence to hop was correlated with the 3 enzyme activities only when the medium contained hop tissue, but not when it contained glucose. This result indicates the importance of glucose in controlling the production of extracellular enzymes.

Thus in summary, this *in vitro* assay for extracellular p.p.o was strongly correlated with ploidy as detected in the present study. Only haploid isolates of *V.dahliae*, and the two haploid isolates of *V.albo-atrum* (measured as described above with DNA Feulgen microdensitometry) tested, exhibited extracellular p.p.o activity in the presence of glucose; all diploid isolates (shown by Feulgen DNA microdensitometry) failed to exhibit detectable extracellular p.p.o. The haploid putative recombinant G22 was the only haploid isolate tested that did not exhibit detectable extracellular p.p.o, giving another indication that this isolate might have been derived via haploidization of the parental diploid strain, G22. Howell (1970) working with artificial heterozygous diploids of *V.dahliae* (sensu stricto Isaac) demonstrated that diploid isolates did not exhibit extracellular p.p.o, an indication that diploidy was suppressing such activities. The same test was used by Horiuchi *et al.* (1990) as an indication of diploidy for the isolates of Group D that were not pathogenic to Solanaceous plants and which had been isolated initially from Cruciferous hosts. Actual measurements of the DNA content of the isolates in the present study confirmed that the extracellular p.p.o was strongly correlated with the ploidy. Thus, this very easy visual test can give a very good preliminary indication of the ploidy.

The extracellular polygalacturonase (pg) activity of all isolates under test here was also studied but this was not correlated to ploidy values. All isolates, irrespective their ploidy, exhibited extracellular pg and thus we conclude that this test can not be used for the differentiation of the isolates of the two groups. Howell (1970) had earlier reported that artificial heterozygous diploids also showed suppressing the pg. activity, but this was not confirmed for the natural diploid isolates under test in the present study.

All the above results obtained using different morphological and enzymatic characters, as well as measurements of DNA content, clearly differentiated the isolates into two main groups:

Isolates of **Group A,** obtained from different **non-Cruciferous** hosts and locations, were **haploid,** produced conidia of **5.5μm or less,** on conidiophores with **4-5 phialides/node,** forming almost **spherical compact microsclerotia** on different media and exhibiting extracellular **p.p.o activity** on a modified medium with tannic acid.

Isolates of **Group B,** obtained from **Cruciferous hosts** from different countries, were **diploid,** produced conidia of **7μm or more,** on **longer** conidiophores with **3-4 phialides/node,** forming **irregular, elongate microsclerotia** on different media, and **failed** to exhibit extracellular p.p.o activity on the above modified medium with tannic acid.

Four putative recombinant strains were detected that were the exception to the above rules and which exhibited intermediate or mixed characters. Isolate **G22** that had been obtained from a diploid parent isolated from rape, was of a haploid type for all the other haploid-associated characters tested, but it did not exhibit detectable extracellular p.p.o activity which normally was a diploid-associated character. Isolate **G19** isolated from rape in Germany in 1989 was shown to be haploid by Feulgen microdensitometry, although it produced long spores in long conidiophores with 3-4 phialides (i.e. diploid-associated characters), exhibited extracellular p.p.o (as all haploids) and produced both types of microsclerotia (M1 and M2) as well as intermediate ones. Isolate **111** isolated from Brussels sprouts (UK, 1957) produced short spores but was shown to be diploid by DNA microdensitometry; it formed two types of microsclerotia and failed to exhibit p.p.o (a diploid-associated character). Isolate **195,** the original diploid Stark isolate (Sweden, 1961) was diploid as judged by all the above associated characters except for the formation of microsclerotia; when a few of these were produced they were not of the elongate type normally typical of diploids.

To verify the above results and to further characterize genetically the isolates of the haploid strains of *V.dahliae* (Group A), the isolates of the diploid Crucifer strain viz. *V.d.longisporum,* as well as the four putative recombinants, their genomic DNA was amplified using 20 different single, short, nucleotide primers by the PCR reaction under low stringency conditions. Two isolates from *V.albo-atrum* (one from lucerne 230 and one isolate 234 from chrysanthemum) were also included in the analysis. Welsh & McClelland (1990) and Williams *et al.* (1990) first introduced this modified PCR reaction [RAPDs technique] and since then, it has been widely used to detect inter- and intra-specific DNA polymorphisms in a variety of fungi including *Verticillium.* In this laboratory, Roberts *et al.* (1994) separated 3 *Verticillium* species by RAPDs i.e. *V.dahliae* , *V.albo-atrum* and *V.lecanii,* and they also reported a high degree of similarity between *V.dahliae* and *V.albo-atrum* as compared with a high degree of dissimilarity between the two plant pathogenic species *V.dahliae, V.albo-atrum* and the entomopathogenic *V.lecanii.* Koike *et al.* (1994) using different OPERON primers (OPA4, OPA10) distinguished *V.albo-atrum* from *V.dahliae* as well as isolates of *V.albo-atrum* from lucerne from the rest of the isolates of *V.albo-atrum* they tested. In the same study using two other primers from OPERON (OP4 and OP10), they also distinguished to a certain extent the 5 pathogenicity groups of *V.dahliae* detected in Japan, including the

Group that consisted of isolates pathogenic to Cruciferous plants, that had been identified according to differential hosts (eggplant, tomato, sweet pepper, and Crucifer hosts). This powerful technique also differentiated races of *F.solani* f.sp. *cucurbitae* (Crowhurst *et al.*, 1991), *F.o.pisi* (Grajal-Martin *et al.*, 1993), *F.o.ciceris* (Kelly *et al.*, 1994), *F.o.dianthi* (Manulis *et al.*, 1994), as well as aggressive from non-aggressive isolates of *Phoma lingam* (Goodwin & Annis, 1991; Schäfer & Wöstemeyer, 1992).

In the present study, only 3 out of the 20 primers used produced informative amplification patterns; one OPERON Inc. (USA) primer (OPA 13) and two primers (P2 and P4a) that had been initially developed by Roberts (1992) to specifically amplify the IGS region of *P.hordei* in this laboratory. One of the above primers (P2) from the IGS region of *P.hordei* had been previously shown in this lab. to discriminate species of *Aspergillus* (J. Madeira, personal communication), as well as pathotypes of *Fusarium oxysporum* f.sp. *ciceris* (Kelly *et al.*, 1994).

Thirty-seven bands (between 0.4kb and 2.35kb) were generated in total with the three primers for all isolates. These bands were reproducible and gave informative data concerning the three different groups (Group A, Group B and putative recombinants), as well as the species (*V.dahliae-V.albo-atrum*) and pathotypes (lucerne and non-lucerne *V.albo-atrum* isolates). Only 10 bands (7 generated from diploid isolates and 3 from haploid isolates) discriminated typical haploid isolates of Group A from the typical diploid isolates of Group B, indicating a close genetical relationship between the two groups. Two bands (0.65kb and 1.05kb) were generated only from diploid isolates using primer P2; 2 bands were generated with primer P4a: one (1.40kb) only from diploid isolates and the other (1.28kb) only from haploid isolates; 6 bands were generated with primer OPA13: four bands (1.55, 1.35, 0.95kb and 0.70kb) only from diploid isolates and two bands (1.22kb and 1.45kb) only from haploid isolates. The five (0.65, 1.40, 1.55, 0.95 and 0.70) out of seven bands generated only from the diploid isolates were also generated by the isolate 230 (*V.albo-atrum* strain from lucerne); however only one of them (band 0.95kb) was generated using the isolate 234 (*V.albo-atrum* strain from chrysanthemum). When DNA from another three isolates of *V.albo-atrum* (one isolate: 220 from lucerne, one fluctuating isolate from hop: FH692, and one progressive isolate from hop: PV3) was amplified with primers P4a and OPA13, only the lucerne isolate of *V.albo-atrum* generated the 1.40 kb and 1.55kb bands that had been only generated by the diploid isolates of *V.dahliae* i.e. *V.d.longisporum*. The other 2 isolates of *V.albo-atrum* tested here generated the 1.28 kb band that only been only generated from the haploid isolates of *V.dahliae*.

There were no bands that were unique to the normal Group A of haploid *V.dahliae* strains. However, the 2 *V.albo-atrum* lucerne isolates tested (230, 220) generated distinct patterns with all primers tested (6 bands unique to this strain); the *V.albo-atrum* isolate from chrysanthemum generated 3 unique bands. The fact that there were 6 unique bands to the specific pathotype of V.albo-*atrum* from lucerne, agrees with all other evidence based on 1) pathogenicity [Heale & Isaac, 1963; Bush & Smith, 1982; Christen & Frenche, 1982, Correll *et al.*, 1988], 2) VCGs [all lucerne strains were in the same VCG01 irrespective of the geographical origin (Correll *et al.*,1988)], 3) mt-RFLPs (Typas *et al.*, 1992), 4) RFLPs [(Carder & Barbara, 1991; Okoli *et al.*, 1994)], 5) sequence of ITS regions [(Morton *et al.*, 1995)]. All these reports indicate that the *V.albo-atrum* lucerne strain is a distinctive *V.albo-atrum* pathotype and with particular associated characters [e.g. optimum growth at 25^0C-26^0C as compared with optimum growth at 22^0C-23^0C for the other strains of *V.albo-atrum* including *V.albo-arum* from chrysanthemum and hop,

(Heale 1985)]. It has been suggested (Griffin, Bainbridge & Heale, in press) that the name *V.albo-atrum* var. *medicaginis* be adopted for this specialized pathotype.

DNA from the two isolates from hop (one progressive and one fluctuating) did not generate distinctive patterns with primer P4a, but gave distinctive patterns with OPA13. Although we used a small number of *V.albo-atrum* isolates (5) here, the data obtained from the RAPDs, suggest that there could be a direct genetic link between diploid isolates of *V.d.longisporum* on the one hand, and haploid isolates of *V.albo-atrum* obtained from lucerne on the other hand as they shared 4 common RAPD bands that were not present in the any of the haploid *V.dahliae* isolates tested. This result give a first indication that one of the possible parents of the diploid *V.d.longisporum* strain could be a *V.albo-atrum* haploid isolate? The suggestion of a close genetic relationship of the diploid isolates of *V.d.longisporum* with isolates of *V.albo-atrum* from lucerne is in agreement with molecular results previously obtained by other authors e.g. Morton *et al.* (1995) who sequenced the ITS1 and ITS2 regions of the ribosomal RNA genes of isolates of *V.dahliae* (haploids) and *V.d.longisporum* (diploids, isolates 161, 162, 84013, 84120), as well as *V.albo-atrum* isolates from lucerne and non-lucerne hosts. These authors, found that the ITS regions of the diploid isolates were identical and were separated from the lucerne pathotype group of *V.albo-atrum* by only one nucleotide, although they were separated from the haploid isolates of *V.dahliae* strains by at least 6 nucleotides. Thus, diploid *V.d.longisporum* isolates, on the ITS-1, ITS-2 sequence basis, were more closely related with lucerne isolates of *V.albo-atrum* than to haploid isolates/strains of *V.dahliae.* Results previously obtained by the same group (Okoli *et al.*, 1994) by RFLP analysis, cluster the diploid isolates (161, 162, 84013, 84120, 86207) in a separate group that was distinct from the haploid isolates of *V.dahliae* and of *V.albo-atrum* isolates; the proportion of polymorphisms which distinguished any of the haploid groups from each other (61%) was slightly lower than the proportion of polymorphisms which distinguished the Group of diploids (*V.d.longisporum*) from the other haploid groups of *V.dahliae* or *V.albo-atrum* (69% at least). Additionally, Morton *et al.* (1995) sequenced the sub-repeats of the ribosomal RNA intergenic regions (IGRs) of haploid isolates of *V.dahliae*, diploid isolates of *V.dahliae* (=*V.d.longisporum*) and haploid isolates *V.albo-atrum* and found them to be organised differently, concluding that all three have to be regarded as separate species. The RAPDs analysis from the present study indicate that *V.dahliae* var. *longisporum* could have evolved as an hybrid between a *V.dahliae* haploid and a *V.albo-atrum* haploid isolate from lucerne because, with all primers tested, the discriminating bands that were generated only from the diploid isolates of *V.d.longisporum* (i.e. those bands which separated isolates of the haploid strains of *V.dahliae* from isolates of the diploid strain *V.d.longisporum*) were also produced by *V.albo-atrum* lucerne pathotype isolates under the same 'RAPDs conditions'.

The putative recombinants, 111, G22, G19 and 195, detected by the other, previously described, ploidy-associated, characters tested, were also confirmed by RAPDs analysis. Isolate 111 generated only five of the 7 discriminating bands produced from the typical diploid isolates (1.55kb, 1.35kb, 1.40kb, 0.95kb and 0.70kb) and none of the 3 discriminating bands generated only from the haploids. Isolate G22 generated two of the discriminating bands (0.65kb, 1.05kb) produced only from the diploid isolates and all 3 (1.28kb, 1.22kb, and 0.45kb) of the discriminating bands generated only from the haploid isolates. Isolates G19 and 195 produced six discriminating bands generated only from the diploids (0.65kb, 1.05kb,

1.40kb, 0.95kb, 0.70kb and 1.55kb) and two of the discriminating bands produced only from haploid isolates (1.28kb and 1.22kb). Two isolates G19 and 195 could not be distinguished from the RAPDs data obtained with the three primers tested, although previously in this study they were found to be different in their ploidy. One band (1.10kb) was only generated with primer P2 from isolate 111, and also with primer P4a from 195 and G19. If the number of 'diploid' or 'recombinant- associated' bands 'missing' in the 8 haploid *V.dahliae* isolates are counted as nine (7 generated from all diploids and 2 generated from recombinants only) the corresponding number of 'diploid' or 'recombinant-associated' bands 'missing' in the two *V.albo-atrum* haploids are also nine. This comparison of course is not entirely fair, as it involves data from just two *V.albo-atrum* haploids compared with the 8 normal *V.dahliae* haploids. However, it is one more piece of evidence suggesting that the *V.albo-atrum* contribution to the percentage of the diploid *V.d.longisporum* genotype may approach that of the *V.dahliae* parent or may at least be a significant contribution.

Cluster analysis derived from UPGMA analysis on RAPDs data separated all the *V.dahliae* isolates into **two distinct clusters**. The first cluster consisted of haploid isolates: the 8 typical haploid isolates of *V.dahliae*, irrespective of the host the came from and the one putative recombinant haploid isolate G22. The second cluster consisted of the 19 isolates of the diploid strain *V.d.longisporum*, plus the two putative recombinants G19 and 195 and the putative recombinant 111 diploid isolate from Brussels Sprouts (UK). These two clusters although distinct were found to have 52% similarity, indicating a close relationship between the two groups. More diversity was found between the haploid strains of *V.dahliae* as compared with that within the diploid isolates of the strain *V.d.longisporum*. This is as expected as isolates of the haploid strain had been isolated from different hosts and locations, whereas isolates of the diploid strain *V.d.longisporum* were mainly from oilseed rape and some Brassica-related species. The sub-groups formed in the main cluster of the diploids had no correlation with the geographical origin of the isolates, except that the two isolates from Sweden (161,162) were identical on this criterion and formed a sub-group inside the cluster of diploids. The other sub-groups formed in the cluster of diploids consisted of isolates from different geographical origins; one geographically diverse sub-group consisted of isolates G34, G334, G25 from Germany, F654-2 from France, P671 from Poland and 86207, 84120 from Japan; nevertheless, they showed 100% similarity by this criterion. Another sub-group consisted of the 195 original diploid isolate of *V.d.longisporum* Stark (1961) and the putative recombinant G19; another subgroup consisted of two isolates from Germany (G29, G23) and 2 isolates from France (F617-9 and F-617-7), and another sub-group consisted of two isolates from Germany (GV167, G859) and one from Japan (84013). Isolate 111, although in the same main sub-cluster of diploids was quite distinct from the other diploid isolates (18%-23% less similar than the other diploid isolates). Thus, the limited molecular variation found among the diploid isolates was not strongly related to geographical origin. No particular character associated with these sub-groups. In the cluster of haploids, isolates from tomato (130, 133) and Shepherd's purse (362) from USA formed a sub-group. Isolates G1, G16 and G22 although placed in the cluster of haploids were less related to the other haploid isolates as expected, isolates of *V.albo-atrum* from lucerne and chrysanthemum were clearly distanced from each other.

All the above data from RAPDs analysis distinguished the isolates of *V.dahliae* in group A (haploids) from those in Group B (diploids), as well as the 4 putative recombinants (G22, G19, 195 and

111). This evidence agreed well with all the previous work that had been reported by other authors using a smaller number of diploid isolates and different molecular biology techniques i.e. RFLPs, sequence data of ITS regions and sub-repeats in IGR. Okoli *et al.* (1994) studied the RFLPs polymorphisms of in the two diploid isolates (161 and 162) from Sweden, the 3 diploid isolates 84120, 86207 and 84013 from Japan (shown in the present study to be diploid by DNA microdensitometry) and another 3 isolates from oilseed rape from Germany and found that these isolates formed a distinct group viz. Group D from two other groups viz. Group A and Group B of *V.dahliae* (haploids), from the Group L (lucerne *V.albo-atrum* isolates), the Group NL (non-Lucerne *V.albo-atrum* isolates) and the group with haploid isolates of *V.dahliae* from mint (Group M). In the same study, two probes also discriminated the two Japanese isolates from Chinese cabbage (84120, 84013) from the other 6 isolates used, including the isolate 86207 also from Japan. Our data from RAPDs did not cluster the two isolates from Chinese cabbage, but the microsclerotia formed on Prune Yeast Lactose medium (Talboys, 1960) by the isolate 86207 were thinner and longer as compared with the above two isolate from Chinese cabbage. Additionally, Carder & Barbara (1994) studied the RFLPs of Japanese isolates previously classified in 4 distinct groups by Horiouchi *et al.* (1990). They studied two isolates of the Japanese Group D (isolates pathogenic only to turnip); isolate 84020 from turnip and 84122 from Chinese cabbage), that were assumed to be related to *V.d.longisporum* in the same study (Horiuchi *et al.*,1990), corresponding with the above RFLP Group D (Okoli *et al.*, 1994). Typas *et al.* (1992) using a rRNA probe for RFLP analysis could not distinguish the three diploid isolates of *V.d.longisporum* used (161, 195 and 84120) from the rest of the haploid isolates of *V.dahliae* and the haploid isolates of *V.albo-atrum*, but they did distinguish them from the other three species studied (*V.tricorpus*, *V.nigrescens* and *V.lecanii*). Sequencing of the ITS regions of diploid isolates of *V.dahliae* (=*V.d.longisporum*) revealed that this group of isolates was also distinct from the haploid isolates (Morton *et al.*, 1995) by at least 6 nucleotides and were only 1 nucleotide distant from the lucerne-*V.albo-atrum* pathotype. Morton *et al.* (1995) also report differences in the sub-repeat sequences in the ribosomal RNA intergenic regions of *V.albo-atrum* , *V.dahliae* and *V.d.longisporum.*

According to morphological, enzymatic and molecular characters, isolates of *V.dahliae* species were classified into three main Groups: Group A of haploid strains, Group B of diploid isolates of *V.d.longisporum* and Group R of the four putative recombinants. The electrophoretic karyotypes of 6 isolates (2 typical haploids: G17 and 140, 1 typical diploid isolate: 161 and 3 of the 4 putative recombinants 195, G22 and 111) were compared by separation of chromosome-length DNA by PGFE-CHEF electrophoresis which revealed 5 (or 6), 4, 9 (or 10), 8 (or 10), 7 and 9 chromosome-length bands respectively. Howlett (1989) revealed karyotypes of 12 and 24 chromosomes in *Phytophthora megasperma* suggesting the occurence of diploid and tetraploid strains. Using the *S.pombe* chromosomes as size standards, the sizes of the Verticillium chromosomes here were found to be between 7.271-0.993 Mbs for the diploids and 7.271-2.188 Mbs for the haploids; a total genome size was estimated at approximately **33.98Mbs** for the **161** Sweden isolate of *V.d.longisporum*; of **30.7Mbs** for the now proven diploid isolate **111** of *V.d.longisporum* from Brussels Sprouts (UK, 1957); of **28.03Mb** for the original **195** isolate of *V.d.longisporum* Stark (Germany, 1961); of **28.38 Mb** for the putative haploid recombinant G22 (Germany, 1987), **12.23Mb** for the haploid isolate 140 (Italy, 1976) and of **26.3Mb** for the isolate **G17** of the haploid strain *V.dahliae* (Germany, 1987). For these estimates, each band was assumed to be formed

by a single chromosome; therefore, they are minimal estimates of genome sizes. Typas & Heale (1980) estimated 0.025-0.030 pg/haploid nucleus of *V.dahliae* that corresponds to 28Mbs/ per haploid genome (Hastie & Heale, 1984). According to the results obtained by PFGE here for the haploid genome of 26.3Mb for isolate G17, and 28.38Mb for the haploid recombinant genome of isolate G22 we infer a similar amount i.e. 0.028 and 0.03 pg of DNA/ haploid nucleus respectively. Haploid isolate 140, did not resolve properly, only 4 very faint bands were visible giving a lower estimation of total genome of 0.013pg of DNA/ haploid nucleus.Thus, our results for the haploid isolates are in very close agreement with the results obtained by Typas & Heale (1980) for the estimation of DNA in Verticillium by microdensitometry. The number of PFGE-resolved chromosome-sized bands was shown to coincide with the number of genetic linkage groups detected in *V.dahliae*; according to genetic (parasexual) analysis, i.e. 5 linkage groups (4 large and one small chromosome that occasionally fails to segregate and remains linked to another chromosome) have been proposed for *V.dahliae* and *V.albo-atrum* (Typas & Heale, 1978; Hastie & Heale, 1984); this is again in agreement with the 5 chromosomes revealed for the haploid isolate G17 by PFGE. On the other hand, diploid isolate 161 revealed at least 9 chromosomes, because we do not exclude the possibility that some bands co-migrated in the gel, and also that some were diffuse and could not be resolved. For example the 6.438 Mb band that was present in all isolates under test, was very bright in this particular isolate (161) and it could be a double(this was also more obvious in another run we performed with the same isolate). The other major problem with the PFGE technique using protoplasts, was the smear of background, fluorescing material in the lanes of samples making the identification of diffused bands difficult. Thus, the number of bands could be 10 for the diploid strain of *V.d.longisporum.*

According to the results obtained by PFGE here for the 'diploid' isolates 161, 111, and 195, the approximate estimates are equivalent to 0.037, 0.033, 0.030 pg/ haploid nucleus or 0.074, 0.066, and 0.060pg as complete diploid genomes respectively. Tolmsoff (1983) measured a value of 0.076 pg/conidial nucleus for the original diploid isolate 195, (Stark) which is higher than the estimates made in this study.

The diploid isolates reported for PFGE (*V.d.longisporum*) had 3 chromosome length bands in common (6.438, 4.972, 4.337) and and haploid isolates (except 140 that did not resolved properly) had 2 chromosome-length bands in common (6.438 Mb and 4.019 Mb). The chromosome-length polymorphisms (CLPs) observed involved the complete size range of chromosomal pattern; reports for other fungi mostly involved low-molecular weight, minichromosomes. The direct cytological observations (Heale *et al.*, 1968) and the genetic linkage map (Typas & Heale 1978), and the 5 different bands obtained by CHEF could lead to the assumption that 5 chromosome are present in the haploid *V.dahliae*. The differences observed in chromosome number and length in the haploid recombinant strain G22 do not constitute *per se* a surprising result, as the same variability had been observed in a number of phytopathogenic fungal species: *Fusarium oxysporum, F. moniliforme, Cladosporium fulvum, Leptosphaeria maculans,* (Cooley & Caten, 1991; Talbot *et al.*, 1991; Taylor *et al.*, 1991; Migheli *et al.*, 1993; Morales *et al.*, 1993). Further, the chromosome band 6.438Mb was present in every strain (except the isolate 140, that did not resolved totally), but this does not exclude the probability that this band does not represent homologous DNA molecules since hybridization studies have not performed. The differences in karyotypes between haploid and diploid isolates are further indications of their ploidy differences and that they may represent different species. The main conclusion is that isolates previously established as diploid appeared by PFGE to have

8-10 chromosomes and to share 3 common chromosome-length bands and a larger haploid genome size was estimated, while isolates established as being haploid had significantly less bands (5 and 7 bands for the haploid G17 and the putative recombinant G22 respectively), sharing 2 common chromosome-length bands, and a smaller haploid genome was estimated, giving additional evidence that the Crucifer-isolates (= *V.d.longisporum*) constitute a distinct variety, or probably a separate species i.e. *V. cruciferarum* sp. nov. (Karapapa *et al.*, 1994). In any case, the haploid and diploid numbers proposed here are only best estimates at the present time, based upon a combination of cytology, genetic linkage maps and PFGE, and may have to be revised in the future when better techniques are available for distinguishing whole chromosomes in fungi.

To associate the above morphological, enzymatic and molecular characters with the pathogenicity, all isolates of the three identified groups, were tested against one winter cultivar of oilseed rape (Cobra, double low). **All 17 isolates of the diploid strain *V.d.longisporum* (=*V. cruciferarum* sp. nov.), Group B, were pathogenic to oilseed rape cv. Cobra; all 9 isolates in the Group A (haploids) were non-pathogenic to oilseed rape. Some overlap occurs between the two groups, in that the four putative recombinants detected previously were slightly virulent (isolates 111, 195, G19) or avirulent (isolate G22).** This overlap indicates that for an isolate to be a successful isolate in attacking Brassicas, it probably requires to acquire most if not all, 2n-associated characters studied here. Thus, isolates (recombinants) carrying less than the optimum number of characters acquired, are avirulent or slightly virulent.

To further support the above results, 3 isolates of the **Group B** (G859, 161, G334) and one isolate of **Group A** (130) were tested against 4 other cvs. of oilseed rape (Envol, Idol, Samourai, Falkon), highly recommended for general use (NIAB,1993). Again the same result was obtained, isolates of the diploid strain *V.d.longisporum* were virulent against all 4 cvs and the isolate 130 from the haploid strain *V.dahliae* from tomato was avirulent to all 4 above cvs tested. Thus, *V.d.longisporum* isolates appear to represent a distinct population from that of strains pathogenic to other hosts, in that only isolates recovered from Cruciferous hosts [with the only exception of the isolate 161 (sugarbeet from Sweden)] were virulent to oilseed rape cultivars. Baig (1991) tested 7 of the isolates we used in this study and which were found to be diploid (the two recombinants: 111, 195, and 5 typical diploids:161,162, 84120,86207, 84013). He found that isolates 161,162, and 86207 were also highly virulent to 8 cultivars of oilseed rape (Dublo, Fido, Fiona, Gulliver, Pasha, Libravo, Cobra and Mikado) while 111 was mildly virulent to only 4 more susceptible cvs of oilseed rape); 195 was avirulent. He also tested two isolates of *V.dahliae* from cotton (171,176) and one isolate of *V.tricorpus* from tomato, one *V.albo-atrum* from tomato and he found them to be non-pathogenic to oilseed rape cvs, while the 3 virulent *V.d.longisporum* isolates 161, 162, 86207 were also virulent to 5 other Cruciferous plants (cabbage, Brussels Sprouts, Chinese cabbage, Swede, Japanese wild radish and *Arabidopsis Landsberg erecta*). The putative recombinant 111 (UK, 1957) was found to be only slightly pathogenic to Brussels Spouts. All this evidence is in total agreement with the present results, i.e. that only diploid isolates are virulent to oilseed rape and related Crucifer plants. The reason that Baig (1991) reported that the two other *V.d.longisporum* isolates from Japan (84120,84013)

were not virulent to oilseed rape cvs and related hosts was probably the 'milder' test he used to assess pathogenicity. As mentioned in the results section here, in the beginning of the experiments, difficulties were found in obtaining symptoms in oilseed rape cvs. This is not surprising, taking into consideration that the disease in the field takes a long time to develop, i.e. it does not appear before the ripening of the plants; this has also been shown to be due to the maturity of the plants, since short-cycle *V.d.longisporum*-inoculated Brassicas only develop symptoms when they ripen (Seidel & Zeise,1990). For this reason, we favoured the pathogen by offering access to the vascular system by the damage caused to the root system by uprooting the plant from the vermiculite and additionally by stressing the plants (cultivation in small pots). The root-dipping system used in this present study to determine the pathogenicity of strains of *V.dahliae* and *V.d.longisporum* was very successful in obtaining symptoms in 15 days, and at the same time it differentiated the two populations of isolates (haploid A/diploid B). Even under these 'favourable' conditions for the pathogen (involving host injury), isolates of the haploid strains of *V.dahliae* did not succeed in causing any symptoms to oilseed rape cvs tested. Zeise (1995) tested 14 isolates obtained from oilseed rape and 16 isolates from different hosts, including linseed and tomato strains, and found that only the isolates from oilseed rape (i.e. *V.d.longisporum*) were virulent to this host cv. Ceres. Furthermore, 6 of the isolates she tested (G10, G18, G19, G22, G25 and G34) were the same isolates studied in the present investigation.

According to the test employed to determine the pathogenicity of diploid Group B (*V.d.longisporum*) isolates to oilseed rape cv. Cobra, it was found that the isolates did not exhibit exactly the same level of virulence to cv. Cobra. Five different sub-groups were formed according to their degrees of virulence to oilseed rape cv. Cobra viz. 7 isolates (3 from Germany, two from France, one from Sennicerande and one from Sweden) were of the highest virulence, 2 from Japan were very virulent, 4 others from Germany were moderately virulent , 2 others (one from France and one from Japan) were of low virulence and 2 others (one from Germany and one from Sweden) were only of very mild virulence. Thus, there was **no correlation of virulence with the geographical origin of the isolate, and also no correlation was found between the sub-groups formed in the cluster of diploids by RAPDs data and those sub-groups formed according to levels of isolate virulence.**

When the 3 virulent *V.d.longisporum* isolates were tested against 4 other cvs of oilseed rape (Idol, Samourai, Envol, Falcon), again different levels of virulence were found. Isolates 161 and G334 were significantly ($p=0.05$) more virulent to cv Samourai than isolate G859. There were also significant differences ($p=0.05$) in the susceptibility/resistance of the 5 cvs against the two isolates 161 and G334. Cultivar Cobra was the most susceptible osr cv. to isolate 161 of all the 5 cvs tested, with significant differences ($p=0.05$) in susceptibility from all the others cvs.

The haploid recombinant G22 was not virulent to cv. Cobra and diploid recombinants G19 and 195 were only slightly virulent (only inducing yellowing of the lower leaves) to the same cv. The other diploid recombinant 111 (Brussels sprouts, 1957, UK) it did not produce enough spores for the root-dipping inoculation, but when this same isolate was tested by Baig (1991), he found it to be slightly virulent to 4 other cvs of oilseed rape and also to Brussels sprouts. Thus, this isolate can be regarded as being of slight virulence to oilseed rape. Zeise (1995) working in Germany (Rostock) tested the diploid *V.d.longisporum* parent isolate G22 (producing long spores of 8.61μm length) from which was derived the

haploid recombinant G22 (4.602±0.061μm), and found it be virulent to oilseed rape cv. Ceres. This is another indication that G22 as studied here is a haploid, avirulent recombinant formed from the parental, virulent, diploid *V.d.longisporum* isolate G22. Zeise (ibid.) also tested the haploid recombinant G19 using the same cv. of oilseed rape: Ceres, and found it to be slightly virulent to this cv. In the same study, she also reported different levels of virulence of the oilseed rape (*V.d.longisporum*) isolates tested to cv. Ceres; when the same 14 isolates from oilseed rape and 16 isolates from other different hosts to tomato and linseed were compared, only isolates from pepper and from cotton induced wilt in tomato, and most isolates from all other hosts except those from oilseed rape induced symptoms in linseed. Horiuchi *et al.* (1990) in Japan found that 27 isolates from Crucifer hosts (18 from Chinese cabbage, 4 from Japanese radish, 3 from Broccoli, 2 from Cabbage and 1 from Turnip), that closely resembled diploid isolates 161,162 and 195, were not pathogenic to Solanaceous hosts (eggplant, tomato and sweet pepper), but were pathogenic to the Cruciferous host, turnip. The original *V.d.longisporum* isolate: 195 from horseradish Hamburg (Stark,1961) had been tested by the Stark (Stark, 1963) and was found to be pathogenic to potato but not to tomato. The same result was obtained by Chang & Eastburn (1994) who tested a strain of *V.dahliae* from horseradish and showed that pepper, tomato and watermelon were asymptomatic hosts for this strain. All these results indicate that tomato is a differentiatial host for the *V.d.longisporum* diploid isolates and strongly support the conclusion that this diploid strain viz. *V.d.longisporum* from Crucifer-hosts is a distinct pathotype, which along with all the genetic, morphological and other characters dealt with above, serve to emphasize its separate species status (= *V.cruciferarum* sp. nov) .

Verticillium species are classified within the Fungi Imperfecti, with no perfect state known as yet. Thus, parasexuality, that has been demonstrated in the laboratory by a number of studies, has been assumed to permit these Imperfect fungi to exchange genetic information in the wild, as an alternative to a conventional sexual recombination system. These distinct Crucifer/Brassica-adapted 'diploid' isolates viz. *V.d.longisporum* (=*V.d.cruciferarum* sp. novo) may be recgarded as evidence of the occurrence of parasexual systems in wild populations of these fungi and that new 'genomes' can arise through 'hybridization' and subsequent somatic recombination. The molecular biology data presented here indicate that this new 'diploid' genome may have arisen from a fusion of two nuclei in an heterokaryon formed between two different haploid genomes, probably one that was from a *V.dahliae* and the other a *V.albo-atrum*, possibly from alfalfa/lucerne. Interestingly in this context, there is one report of an alfalfa/lucerne strain of *V.albo-atrum* colonizing a cv. of oilseed rape (Bush & Smith, 1982), although this was achieved by experimental artificial inoculation conditions.

Further, converting all data obtained from all qualitative and quantitative characters and RAPDs into numerical quantities, numerical taxonomy, (UPGMA analysis and co-ordinate analysis) was applied to classify the isolates under test and these were again clearly clustered into 3 main groups: The Group A of haploid strains of *V.dahliae*, the Group B of isolates the diploid species of *V.d.longisporum* (*V.cruciferarum sp. nov.*, Karapapa *et al.*, 1994) and the group of the four recombinants which we conclude have been derived subsequently from *V.d.longisporum*. The two isolates of *V.albo-atrum* tested were not in any of these clusters and were again distinct from each other. A key feature of the numerical taxonomic methods, including the two methods mentioned above, is that *a priori*, all the characters have equal importance and are assumed to be independent. Since we do not know the origin of the bands generated by

PCR amplification under low strigency conditions, it is assumed that the bands produced by RAPDs (PCR amplification with single oligonucleotide primers) were independent characters although this may not always be the case.

The number of differential characters (spore length, conidiophore and microsclerotia formation, ploidy, extracellular p.p.o activity, pathogenicity to oilseed rape and other non-Crucifer hosts, RAPDs, CLPs, RFLPs, sequence data of ITS and IGR) between strains of *V.dahliae* and isolates of *V.d.longisporum* strongly indicates that these latter group of isolates belong to different species and we have proposed tentatively the name *Verticillium cruciferarum* sp. novo (Karapapa *et al.*, 1994). The concept of a species, is that of a group of individuals which can share a common pool of genes through crossing and at the same time are isolated genetically from other groups. In the Fungi Imperfecti, this concept is more artificial than in 'perfect' fungi, because of the lack of a sexual stage (Hastie, 1978). To establish further that diploid *V.dahliae longisporum* constitutes a different species than *V.dahliae* also requires data on compatibility, but this is not yet available, because these isolates of *V.d.longisporum* are 'diploid' and it is therefore very difficult to obtain nit- or colour-deficiency mutants for compatibility studies. The strong evidence on speciation and the origin of this new species, is that this group of 'diploid' isolates are capable of inducing symptoms and disease in oilseed rape cvs and other Brassica-related species, and not on other hosts; thus they live in an 'adapted environment', in a way, isolated from strains of *V.albo-atrum* and *V.dahliae*, i.e. there may be a barrier limiting genetic exchange and conserving speciation. Thus, these populations of 'diploids' have established an isolating mechanism during the active growth of the organism. Verticillium plant pathogens spp. are normally dormant in soil and survive only by their resting structures (Heale 1988). All the above characters tested have value and the proposal to raise the taxonomic position of *V.d.longisporum* to a higher specific rank was based firstly by adding all the characters together, and secondly taking into consideration that using the morphological characters, especially the conidial length size (also the type of microsclerotium), and the enzymatic, visual test of extracellular p.p.o activity, provides a very **convenient, simple and practical tool** for the initial classification of isolates of this fungus. For complete certainty, DNA determination and RAPDs fingerprinting will be required for final identification.

Role of the myrosinase-glucosinolate system of oilseed rape as a defence mechanism against a haploid isolate a of non-pathogenic strain of *V.dahliae*, as compared with infection caused by a highly virulent isolate of the diploid strain *V.d.longisporum* (= *V.cruciferarum* sp. novo).

To investigate the role of the myrosinase-glucosinolate system of oilseed rape against *V.dahliae*, the host-parasite responses of one cultivar (Cobra) of oilseed rape to one haploid isolate (130) of a strain of *V.dahliae* from tomato (non-pathogenic to oilseed rape), and one virulent isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum* (=*V.cruciferarum* sp. novo), [selected from the two Groups (Group A, Group B) as above, as typical representatives], were studied.

The reason that in this investigation one diploid isolate (161) of the Group B and one haploid isolate (130) of the Group A were employed here, is that it was thought that the myrosinase-glucosinolate system acts as a part of the general defence system protecting the plant against insect pests and fungal diseases (Chew, 1988), i.e. not being specific. Furthermore, since 28 isolates of the diploid strain of *V.d.longisporum* (=*V.cruciferarum* sp. novo) were found to be highly virulent to cv. Cobra (and in addition 3 of them : 161, G334, G859, were also highly virulent to 4 other different oilseed rape cvs.) in this study, it was assumed that as a successful invader, this chosen isolate would have overcome this mechanism of host-defence (as also for the other 2 highly virulent isolates). Thus, the role of the myrosinase-glucosinolate system could be investigated to show how this diploid (161), host-adapted pathogen suppressed, or 'avoided' this system of defence to become a successful pathogen, as compared with the haploid, avirulent isolate (130).

Total and individual glucosinolate profiles and glucosinolate concentrations in oilseed rape plants depend upon the genotype (cultivar /variety), cultivation, climate, stresses, as well as tissue from which they are extracted and the developmental stage of the plant. Thus, to investigate the glucosinolate levels of inoculated ('161'-, and '130-inoculated' plants) and control, healthy, oilseed rape plants, we investigated the glucosinolate profiles of roots, hypocotyls, cotyledons and leaves separately at 3, 10 and 15 days post-inoculation by HPLC analysis. Plants (cv. Cobra) had been inoculated by root-dipping when they were two weeks old, thus they were sampled when they were 17, 24 and 29 days old.

In roots of oilseed rape plants, 7 glucosinolates were detected, one aliphatic: **progoitrin (PRG)**, one non-indole, aromatic: **gluconasturtiin (GLNS)**; 4 indole-aromatic glucosinolates: **glucobrassicin (GLBR), neoglucobrassicin (NGLBR), hydroxyglucobrassicin (4-OH-3ind)** and **methoxy-glucobrassicin (4-OH-3ind)**; and one unidentified peak **(GL-1)**, at 3, 10 and 15 days post-inoculation. Previous authors working with other Brasicaceae plants, including different cvs of oilseed rape, also detected the same 4 (above) indole glucosinolates and the aromatic glucosinolate GLNS. Similarly, Sang *et al.* (1984) studied the glucosinolate profiles of oilseed rape cv. Midas by HPLC and they were found to contain PRG in small amounts, the 4 indole-glucosinolates mentioned above and the aromatic glucosinolate GLNS, which was the predominant glucosinolate; among the indole glucocosinolates, GLBR was found to be predominant. Additionally, MacGregor (1988) reported GLNS to be the predominant glucosinolate in roots of oilseed rape cv. Midas; its relative amount increased with development.

In this study, for all inoculated ('130-', '161-inoculated') and control uninoculated **roots**, the individual aliphatic glucosinolate, PRG, was found in similar levels in roots and its concentration decreased over the growth period examined. Differences were detected: i) in levels of the aromatic glucosinolate GLNS and ii) in levels of indole glucosinolates among the three treatments. In roots of control and '130-inoculated' oilseed rape plants, NGLB was the predominant glucosinolate at 3 days post-inoculation; it then decreased during the development of the plant, and GLNS was the predominant glucosinolate at 15 days post-inoculation. Baig (1991) studied the individual glucosinolates in different control healthy oilseed rape cvs and also found that NGLB was the predominant glucosinolate in roots of cv. Cobra at 14 days. In roots of '161-inoculated' plants, NGLB was the predominant glucosinolate at 3, 10 and 15 days post-inoculation and GLNS was detected at reduced levels, as compared with control and '130-inoculated' plants, (it was reduced by 100%, 65% and 85% as compared with the roots of control, uninoculated plants at 3, 10 and 15 days post-inoculation respectively). On the contrary, the same glucosinolate (GLNS) increased 76% in roots of '130-inoculated' plants at 15 days post-inoculation, as compared with roots of control, uninoculated plants. When the data were expressed as percentages of aliphatic, aromatic non-indole, and aromatic indole glucosinolates of the total amount of glucosinolates, an interesting result was revealed. The proportion (% total) of indole glucosinolates (all four together) in roots of '161-inoculated' plants (compatible interaction) was high and was the dominant proportion for all days after inoculation. On the contrary, in the controls and '130-inoculated' plants (incompatible interaction), the proportion of indole glucosinolates decreased over time and the aromatic glucosinolate GLNS accounted for the highest proportion at 10 and 15 days post-inoculation for control plants, and at 15 days post-inoculation for '130-inoculated' plants. MacGregor (1988) studied the glucosinolates in rapeseed seedlings of cv. Midas and reported that in roots, the relative amount of GLNS increased during development and the relative amount of the other glucosinolates decreased, which agrees with the observation here for control plants and plants of the incompatible (isolate 130) interaction. In addition, by 15 days post-inoculation, the amount of GLNS was increased for the incompatible interaction (isolate 130) as compared with the control. This suggests that GLNS synthesis had been inhibited/suppressed in '161-inoculated' roots i.e. the compatible interaction. Thus, in roots, a marked difference in glucosinolates was found between the compatible interaction involving the cv. Cobra and the diploid isolate 161 of the pathogenic strain viz. *V.d.longisporum* on the one hand, and the incompatible interaction involving the same cv. and the haploid isolate of a strain (tomato) of *V.dahliae*, especially at 15 days post-inoculation. In the incompatible interaction (130), there was an increase in the levels of GLNS, thus the major proportion (% total of glucosinolates) was accounted for by this non-indolic, aromatic, glucosinolate; whereas, in the compatible interaction (161), there was a decrease in the levels of GLNS leading to an increased proportion of the aromatic, indole glucosinolates. Previous reports on Brassica infestation with the turnip root fly larvae (*Delia floralis*) and their feeding on the roots of range of Brassica species, showed a significant increase in the relative **proportion** of indole glucosinolates (% total) in the host tissues, as well as the total glucosinolate content (Birch *et al.*, 1990, Griffiths *et al.*, 1994). In the present study, the total glucosinolate content increased by almost 50% only for the incompatible interaction (130) at 15 days post-inoculation.

Three of the four indole-glucosinolates showed increased levels in roots of '161-inoculated' plants at only 3 days post-inoculation (26% increase in total indole glucosinolates, as compared with the control, uninoculated roots). GLBR increased 88%, NGLBR by 12% and 4-MeO-3ind by 100%, as compared with control, uninoculated roots. This is in agreement with previous studies with Brassica pests and pathogens, as well as damage (artificial wounding) which showed that indole-glucosinolates were induced. Birch *et al.* (1990) found that NGLBR increased by over 88% in roots of infested winter oilseed rape plants with the turnip root fly *Delia floralis*. Additionally, Butcher *et al.* (1974) studied the levels of glucosinolates in roots and stems of *Brassica rapa* after infection with *Plasmodiophora brassicae* (club root) and found GLBR levels to be markedly increased (doubled during the growth of 'clubs'). Similarly, Koritsas *et al.* (1991) found increases in the indole NGLBR in roots of oilseed rape plants infested with cabbage stem beetles (*Psylliodes chrysocephala*), while the aliphatic and aromatic compounds declined. In the same study, they reported that the major compound in root tissue from non-infested plants was the aromatic glucosinolate GLNS.

In the present study, the levels of the aromatic, **non-indole**, glucosinolate GLNS as compared with these of the aromatic **indole** glucosinolates, showed differences after inoculation of oilseed rape according to which isolates (161 or 130) were employed, i.e. in a compatible as compared with an incompatible interaction respectively, and this is consistent with the different biosynthetic pathways that these two major types of glucosinolates are derived from. It is well established that GLNS derives from phenylalanine, whereas indole glucosinolates form from tryptophane (Underhill, 1980). Here, biosynthesis of indole glucosinolates was induced initially and they were in the highest proportion for all days post-inoculation tested, due apparently to the suppression of gluconasturtiin synthesis in the compatible (161) interaction. On the contrary, **GLNS increased in the incompatible (130) interaction** to levels that increased the proportion of aromatic glucosinolates to the high levels that were found in the control healthy plants. This result supports previous data obtained by Baig (1991) who performed bioassays examining the effect of glucosinolate/myrosinase breakdown products on different isolates of *V.dahliae* and *V.d.longisporum;* he found that gluconasturtiin (GLNS) alone at high concentrations produced a mild reduction in fungal growth, but more importantly, that its breakdown products (as compared with breakdown products of other glucosinolates) had the **most pronounced inhibitory effects** on growth of the isolates. The pathogenic group of diploid *V.d.longisporum* isolates he tested (including the isolate 161 as used here) were **much less sensitive** than the non-pathogenic group (haploid isolates) to **GLNS**- and also to **GLNP**-breakdown products. This implies a certain adaptation in the diploid *V.d.longisporum* to this general defence mechanism in Brassicas. Thus, the diploids (i.e. *V.d.longisporum*) as part of a process of achieving basic compatibility, are less sensitive to this non-specific deterrent, i.e. enzymic breakdown products of glucosinolates, more especially GLNS and GLNP, than is the case of the non-pathogenic, haploid, *V.dahliae* strains. At the same time, the present results indicate a **suppression of the biosynthesis of this aromatic glucosinolate (GLNS)**, in the compatible interaction (161), by comparison with levels in the incompatible (130) interaction. Furthermore, indole glucosinolates which increased in the compatible interaction (161), are known to produce nitrile derivatives i.e. glucobrassicin; the latter produces 3-indolyl-acetonitrile,which following nitrilase activity, can produce indolylacetic acid (IAA) [Searle *et al.*, 1982], which is known to increased in vascular wilt diseases (Beckman, 1987).

In hypocotyls, 3 aliphatic glucosinolates: PRG, NPL and GLNP were detected: the aromatic non-indole glucosinolate: GLNS; 3 aromatic indole glucosinolates (GLBR, NGLBR and 4-MeO-3ind) and 3 unidentified peaks. Griffiths *et al.* (1994) found increased proportions of indole glucosinolates in stems of larval (*Delia floralis*) damaged roots which agrees with the results obtained in this study for the hypocotyls. Further, Koritsas *et al.* (1989) reported that infestation of stems of oilseed rape with *Psilliodes chrysochephalla* (cabbage stem flea beetle) led to an increase in indole glucosinolates and especially GLBR. The composition of the three aliphatic glucosinolates in the present study, was the same for all treatments, and these aliphatic glucosinolates accounted for the major proportion of the glucosinolate content of hypocotyls for all treatments at 3 days post-inoculation. At 10 days post-inoculation, indole glucosinolates accounted for the major proportion for all treatments, with the highest percentage found in '161-inoculated' plants (compatible interaction), **probably due to GLNS being suppressed in '161-inoculated' plants.** At the same time, the aliphatic glucosinolate: GLNP was found in decreased levels in the compatible (161) interaction, thus decreasing the proportion of aliphatic glucosinolates (50% less, as compared with the control, and '130-inoculated', plants). At 15 days post-inoculation, the same phenomenon was observed; the aromatic glucosinolate **GLNS was undetectable in '161-inoculated plants' (compatible interaction)** and indole glucosinolates accounted for the largest proportion of the composition of glucosinolates in hypocotyls of these plants. On the contrary, in hypocotyls of control, and '130-inoculated', plants (incompatible interaction), **the major compound was GLNS.**

These results for both roots and hypocotyls, suggest that in the incompatible (161) interaction, the production of the aromatic no-indole glucosinolate GLNS is strongly inhibited/suppressed.

In cotyledons, two aliphatic glucosinolates were detected: PRG, GLNP; four aromatic, indole glucosinolates: GLBR, NGLBR, 4-MeO-3ind and 4-OH-3ind, and the aromatic, non-indole glucosinolate: GLNS. Additionally, one unidentified peak was observed. GLBR was the predominant glucosinolate for all days and treatments. Bodnaryk (1992) reported the same major glucosinolate (GLBR) in cotyledons of *Brassica napus* cv. Westar, as well as in cotyledons of two other Brassica species: *B.rapa* and *B.juncea*. In the present study, the total glucosinolate content remained unchanged in cotyledons of healthy control plants, but changed considerably in infected cotyledons (161), especially at 10 days post-inoculation, increasing 50%, due to the large increase in the amount of GLBR (295% increase as compared with that of control healthy cotyledons). A similar observation was reported for cotyledons of oilseed rape (cv.Westar) and *B.rapa* and *B.juncea*, after wounding with a needle or feeding by the adult flee beetle, *Phyllotreta cruciferae* where increases of up to 359% were reported (Bodnaryk, 1992). **The opposite result for the aromatic non-indole glucosinolate GLNS and the aliphatic glucosinolate GLNP was observed in cotyledons, as compared with roots and hypocotyls, in the present study; GLNS and GLNP increased considerably in '161 infected' (compatible interaction) cotyledons at 15 days post-inoculation, as compared with the other treatments.** Peterka &Schlosser (1989) demonstrated the correlation between sinigrin and GLNS content in cotyledons in *B.napus* and *B.juncea* and resistance to *Leptosphaeria maculans*, varieties of the former species, with lower levels of GLNS in their cotyledons as compared with a *B.juncea* variety, were less resistant to the pathogen.

In leaves ,the same glucosinolates were detected as in cotyledons with the addition of 3 more unidentified peaks. The total glucosinolate content decreased with age for all treatments. Indole glucosinolates were in the highest proportion and GLBR was the predominant glucosinolate for all treatments and days post-inoculation. Baig (1991) reported the same major glucosinolate: GLBR in leaves of 2 week-old oilseed rape plants, cv. Cobra. Exceptions were the leaves of the incompatible (130) interaction, where **an increase in aliphatic glucosinolates** was observed and indole glucosinolates decreased, although the total amount of glucosinolates was the same for all treatments. Doughty *et al.* (1991) reported a variable response (the response differed between the cvs and between leaves of different ages) of these aliphatic glucosinolates in osr cvs. Bienvenu and Cobra after infection with the fungus *Alternaria brassicae*. In the present study, in leaves of plants infected with isolate 161, (compatible interaction) an increase of 4MeO-3ind was observed (168%) at 10 days post-inoculation, thus increasing the total amount of glucosinolates (35%), as compared with the control leaves. As also observed with cotyledons, GLNS increased in leaves of '161-infected' plants (compatible interaction) at 10, and also at 15 days, post-inoculation. Thus, GLNS levels in cotyledons and leaves responded differently to inoculation, as compared with the response of hypocotyls and roots. **In the former organs (cotyledons and leaves) there was an induction of the aromatic glucosinolate (GLNS)** after 15 (for cotyledons) and 10 and 15 (for leaves) days post-inoculation. Additionally, GLNP levels were increased in cotyledons for the compatible interaction (161).

In summary,

1) Total glucosinolate content increased in the **incompatible interaction** involving the isolate 130 of the non-pathogenic strain *V.dahliae* (tomato) and the cv. Cobra (double-low) in **roots, cotyledons, hypocotyls** and **leaves** at 15 days post-inoculation, as compared with healthy controls and plants inoculated with the virulent isolate (161) of the pathogenic diploid strain *V.d.longisporum* (compatible interaction)

2) The levels of the aromatic, non-indole glucosinolate :**GLNS** increased during development in **roots** and **hypocotyls** of control, and '130-inoculated', plants (incompatible interaction), and accounted for the majority of the composition of glucosinolates in these tissues. In '130-inoculated' plants (incompatible interaction) GLNS levels were higher than the levels in the control, healthy plants. On the contrary, in the **compatible (161) interaction**, aromatic, indole glucosinolates accounted for the major percentage of glucosinolate composition and **biosynthesis of the aromatic, non-indole glucosinolate: GLNS was inhibited/suppressed.**

3) The total glucosinolate content **in cotyledons** did not change considerably during the development of the plant in healthy control, or '130-inoculated', plants (incompatible interaction). On the contrary, the **total glucosinolate** content changed considerably over time after inovulation in the '161 inoculated' plants' (compatible interaction); e.g. increasing by 50% at 10 days post inoculation, due to the major increase in the indole glucosinolate: GLBR. **GLBR** was the predominant glucosinolate in both (130, 161) interactions as well as in control healthy plants.

4) The concentration of the aromatic, non-indole glucosinolate: **GLNS** increased in '161-inoculated' infected **cotyledons** (compatible interaction), **at a late phase in colonization and the onset of severe symptoms** i.e. at **15 days** post-inoculation, as well as in **leaves at 10 and 15 days** post-

inoculation; **this is the reverse result of what was observed in hypocotyls and roots in the same treatments.**

5) The levels of the aliphatic glucosinolate **GLNP decreased in the hypocotyls** of the compatible interaction (161), although it **increased in cotyledons** of the same interaction .

6) Aliphatic glucosinolates found in increased levels in cotyledons and leaves in '130-inoculated' plants at 3 and 10 days post-inoculation respectively, but this was not observed for '161-inoculated" (compatible interaction) plants.

For all these results, it appears that increased **GLNS** biosynthesis in **roots** and **hypocotyls** is probably a major factor in the non-specific, active resistance of o.s.r. against non-pathogenic strains of *V.dahliae*, which are unlikely to colonize the plant further. Compatible strains (*V.d.longisporum=V. cruciferarum* sp. novo) appear to suppress GLNS synthesis in roots, or avoid triggering its synthesis, perhaps by avoiding recognition? **At a later stage of the infection,** when the successful pathogen (*V.d.longisporum= V.cruciferarum* sp. novo) reaches the upper shoot and leaves (10 and 15 days post-inoculation in the seedling/root dipping test employed here), there is a major increase in cotyledons and leaves of the aromatic, non-indolic, glucosinolate: GLNS in the compatible/susceptible interaction, probably due to the **non-specific triggering of non-indolic aromatic glucosinolate biosynthesis in adjacent healthy tissue after cellular necrosis** is caused by toxins/enzymes (Beckman, 1987) secreted by the compatible fungus in advance of its growth. There was also some evidence for a systemic response of cotyledons and leaves in the incompatible interaction via an increase in aliphatic glucosinolates.

Myrosinase assays were also performed at 3, 10 and 15 days post-inoculation, using enzyme extractions from roots, hypocotyls, cotyledons and leaves. All assays were conducted with desalted extracts in the presence of ascorbic acid that is well known to activate the enzyme (Tsuruo & Hata 1968), thus assuming that the 'potential' activity was measured (James & Rossiter, 1991). Myrosinase activities were measured by the determination of the released glucose using a specific glucose kit (Sigma). In control extracts, the specific myrosinase activity was increased during the time course study. In roots, a large initial increase in myrosinase activity was found for both compatible (161) and incompatible (130) interactions, as compared with the specific activity of extracts of control, uninoculated healthy plants, indicating that this is a general reaction of the host following attempted invasion of both strains. At 10 and 15 days post-inoculation, no major differences were found in the specific activity root extracts among all treatments.

A very high specific myrosinase activity was detected at 10 days post-inoculation in hypocotyl extracts in the incompatible (130) interaction, as compared with control extracts. However, the amount of total protein in these extracts was considerable smaller, suggesting that the activity in hypocotyls in the incompatible interaction resulted from **activation, rather than de novo synthesis. On the contrary, extracts of hypocotyls of '161-inoculated' plants showed a decreased specific myrosinase activity,** although the amount of total protein was very high. This may be a result of recognition of a non-pathogenic strain, resulting in enhanced myrosinase activity levels, and to the accumulation of glucosinolate breakdown products to toxic levels that lead to a hostile environment, thus inhibiting further development of the non-pathogenic haploid strain of *V.dahliae*. Increased levels in myrosinase activity in hypocotyls of OSR cv. Cobra in the incompatible (130) interaction at 10 days post-

inoculation, was also associated with increased levels of the aliphatic glucosinolate: GLNP and the aromatic, non-indolic, glucosinolate: GLNS, whose hydrolysis products had previously been shown to be toxic to fungal growth (i.e. *V.dahliae*) in this laboratory (Baig, 1991). Further, when colonization studies were conducted for both '130-', and '161-inoculated, plants', **the avirulent isolate (130) of the non-**pathogenic, haploid strain *V.dahliae* **was still recovered from the hypocotyls up to 15 days post-inoculation after which the fungus was not recoverable**, suggesting that the 130 isolate had in fact been completely killed by this time. The apparent decrease in colonization with time that was observed earlier by 9 days post-inoculation, may therefore reflect the gradual death of fungal hyphae caused by increasing host release of antifungal compounds, i.e. the toxic products of the enzymic breakdown of GLNS and GLNP. At 10 days post-inoculation, decreased levels in myrosinase activity were found in hypocotyl extracts of '161-inoculated' plants (compatible interaction), as compared with both uninoculated healthy controls and '130-inoculated' (incompatibel interaction) plants; this was also associated with decreased levels of GLNS and GLNP discusseed earlier. From colonization studies, **the diploid isolate (161) of the pathogenic diploid strain viz. *V.d.longisporum* showed a steady increase in systemic colonization (compatible interaction) and the fungus was recoverable at all days tested from the hypocotyls**; by the 9th day it had been recovered from all segments and by the 15th day post-inoculation the plant was 100% colonized.

Thus, in incompatible (130) and compatible (161) interactions, the host plant reacts differently. In the incompatible (130) interaction, the plant appears to recognise the non-pathogen and this results in increased levels of GLNS and GLNP combined with enhanced myrosinase activity, followed by the effective inhibition of the fungus. In the compatible interaction (161), the host plant apparently fails to recognise the virulent isolate of the diploid pathogenic strain of *V.d.longisporum* (=*V.d.cruciferarum* sp. novo). It is possible that the pathogen produces a suppressor that blocks the active synthesis of GLNS and GLNP. These suggestions are also supported by the results previously obtained by Baig in this laboratory (1991) who found that isolates of the diploid strain *V.d.longisporum* (including the 161 isolate that we used in this study) were relatively tolerant to GLNS- and GLNP-breakdown products, as compared with avirulent, haploid isolates.

As far as we can ascertain, there are no other reports in the literature of induced levels of myrosinase activity followed infection or artificial damage in Brassica plants. Visvalingam *et al.* (1995) studied the effect of cotyledon wounding on the myrosinase enzyme system in *S. alba* seedlings, and found no marked responses in myrosinase activity in extracts from cotyledons and roots/stems between plants of unwounded and wounded cotyledons. In the present study, cotyledons and leaves showed no marked changes in the specific activity of the enzyme myrosinase between the treatments, and its activity decreased during the development of the seedling. When myrosinase assays were conducted using extracts of petioles of leaves, a large increase was found in extracts of infected petioles of plants inoculated with the isolate 161 (compatible interaction) of the diploid pathogenic strain *V.d.longisporum* at 20 days post-inoculation. **The extractions were performed from tissues that showed symptoms of infection (chlorosis/necrosis) and also microscopic examination revealed that the fungus had colonized the vessels of these petioles, suggesting that the late increase in myrosinase activity is a result of triggering of non-specific defence mechanisms in healthy tissue surrounding necrotic cells. Thus, in**

this case, increased levels in activity appears to occur as a late result of successful tissue colonization in the compatible (161) interaction.

Using different techniques (morphological, histochemical, cytochemical, cell fractionation, immunocytochemical), previous authors have reported the localization of the enzyme myrosinase, in either specific cells (idioplasts, myrosin cells), or in unspecific cells in tissues of different Brassicaceae species; it was found to be either membrane-associated in unspecific cells, or non-membrane-associated in vacuoles of specific cells. Recent immunocytological techniques (immunofluorescence and immunogold labelling) using specific antibodies raised against the enzyme have shown that the enzyme is in fact localized in specific cells (myrosin cells) [Thangstad *et al.*, 1990; Thangstad *et al.*, 1991; Höglund *et al.*, 1991 and Höglund *et al.*, 1992]. Even though both these groups localized the enzyme in specific cells, immunofluorescence showed that myrosinase was membrane- associated while **immunogold labelling revealed that myrosinase was inside the myrosin grains in myrosin cells** which were **distributed in embryos, radicles and cotyledons of *B.napus*, *S.alba* and *R.sativus* tissues.**

In the first attempts to localize the enzyme myrosinase *in situ* in the present study, sinigrin , barium chloride and ascorbate were used in the incubation solution with hand-cut sections of radicles of oilseed rape and also *R. sativus* (radish). This resulted in black deposits which were assumed to be barium sulphate, the hydrolysis product of sinigrin by the endogenous enzyme myrosinase. This histochemical technique **localized the activity of the enzyme in the outer cortex of the radicle**, in the same set of cells that stained intensively blue with lactophenol/cotton blue in the present study. A number of previous studies involving the use of different stains (Millons reagent, lactophenol aniline blue, toluidine blue, methylene azure-II and fuchsin) resulted in the detection of cells in young radicles and cotyledons that stained intensively and specifically. This histochemical technique involving the use of the barium chloride, could not be used further in our studies, due to the difficulty in obtaining hand-cut sections of radicles older than 72 h (the tissue was very fragile and very difficult to obtain hand-cut sections without seriously damaging the cells); additionally myrosinase levels decreased during growth in the healthy uninoculated, controls, thus making it very difficult to localize myrosinase activity with the resolving power of the light microscope.

To further investigate the subcellular localization of myrosinase in the incompatible (130) and compatible interaction (161), we used the powerful technique of **immunogold labelling**. Two different polyclonal antibodies were used: one antibody, viz. M1, was raised against myrosinase I from *Brassica napus* seedlings (diameric 156kD glycosylated protein) by Dr. D. James (Wye College) [James & Rossiter, 1991], and the other antibody, viz. K 089, was raised against myrosinase from seeds of *Sinapis alba* by Prof. A. Bones (Unigen, University of Trondheim, Norway) [Thangstad *et al.*, 1991]. The localization of myrosinase *in situ* was investigated using these antibodies in different tissues and organs of the oilseed rape cv. Cobra (radicles, cotyledons, roots, hypocotyls and petioles) in plants inoculated with either the virulent isolate (161) of the diploid pathogenic strain *V.d.longisporum*, or the avirulent isolate (130) of the haploid strain of *V.dahliae* (tomato), as well as in healthy, control, plants. **All studies at the subcellular level with both antibodies,** showed that the enzyme was localized inside myrosin grains, in specific (myrosin cells), in radicles, cotyledons, hypocotyls and petioles, as well as inside the vacuoles in the **root cap cells and the outer cortex of the root tip. Using this immunogold labelling technique, the**

enzyme was not found in nonspecific cells (idioplasts) and it was not membrane-associated. Preliminary experiments were conducted in Norway in the lab of Prof. A. Bones (Univ. of Trondheim), using LR white semi-thin sections and the immunofluorescent technique revealed the same result, i.e. **the enzyme was localized inside grains of specific cells of radicles or hypocotyls.** When LR-White semi-thin sections of radicles of oilseed rape were stained with 1% toluidine blue, a number of cells in the embryonic axis stained intensively. These areas were selected and when thin sections were treated with the gold-labelled, polyclonal antibodies, the **myrosinase was again located in the same (myrosin) cells that previously had stained intensively blue with toluidine blue i.e. inside the cell, in grains viz. myrosin grains. No labelling was found in membranes, cytoplasm, ER, or dilated cisternae of ER or other organelles of these specific myrosin cells.** These results are in accordance with previous results obtained by Thangstad *et al.* (1991) using the same K089 antibody, and employing radicles and cotyledons of four different Brassicaceae species (*B.napus, S. alba, R. sativus* and *B.oleraceae*). **In the present study, radicles of inoculated plants showed the same positive reaction, and again myrosin grains were shown to be the site of myrosinase.** Unfortunately, no spores or hyphae of the pathogen were detected in tissues of these plants, inoculated with either the virulent isolate (161) or the avirulent isolate (130). The main reason we used the radicles in these tests was to standardise the experimental method (the dilutions of the first and secondary antibodies), as young tissue was known to contain more enzyme and therefore it was easier to localize the enzyme *in situ.*

To investigate the role of myrosinase in host parasite responses involving *V. dahliae* and *V.d.longisporum* ,we first examined the root tips of oilseed rape cv. Cobra at 3, 10 and 15 days post-inoculation, as this is likely to be the first site of entrance of the fungus to the host. **Observations on the localization of the enzyme myrosinase in root tips, made for the first time in this study, showed that the enzyme was localized inside the vacuoles of root cap cells and cells of the outer most cortical area of the root tip, and it was always associated with e-opaque areas and bodies inside the vacuoles. No** such cells with myrosinase-accumulated bodies were observed in the stele, epidermal cells, or in undifferentiated cells that occupied with very small vacuoles. **Conventional staining of the same tissue revealed that these myrosinase-accumulated bodies (mab) that located in the vacuole were encapsulated by a membrane, that separated them from the remain vacuole sap. Thus, these observations provide good evidence for the subcellular organization of the enzyme myrosinase: although is located in the vacuole, it is separated from the other components in the vacuole sap by a membrane.** Previous results obtained by other authors (Grob & Matile, 1979; Helmlinger *et al.*, 1983) using vacuolar fractions and vacuolar markers localized glucosinolates (SNG, GLNS and GLB) inside vacuoles of horse radish root cells. Additionally Grob & Matile (ibid) detected myrosinase activity in the same vacuolar fractions. This 'sub-subcellular' compartmentalization of myrosinase-glucosinolates as supported by the results presented in this study, allows substrate and enzyme components to be separated and consequently 'latent'; thus, when damage occurs, either caused physically or by the presence of a microbial agent, the enzyme myrosinase that occurs in the myrosin bodies is activated when it comes in contact with the substrate glucosinolates. Grob & Matile (1980) detected L-ascorbic acid in vacuoles of horse radish root cells using isolated vacuoles and vacuolar markers; glucosinolates in the presence of ascorbate and myrosinase are known to be hydrolysed after cell rupture, resulting in toxic products i.e.

isothiocyanates (Fenwick *et al.*1983). Previous studies on the localization of glucosinolates in root tips (Wei *et al.*, 1981), using dry-cut cryosections of frozen roots, revealed glucosinolates by detecting the presence of sulphur and showed that these compounds were localized in vacuoles of the root cap cells and root cortex cells. Other parenchymatous cells of the stele and cortex cells contained low amounts of sulphur, indicating a low glucosinolate content.

The results presented in this present study are in accordance with the observations discussed above implying that **the myrosinase and glucosinolates are present in the same compartment (i.e. vacuole) in the same root cap and outer root cortex cells in the root tip, and that they are compartmentalized by a membrane that separates the myrosinase accumulated bodies from the rest of the vacuolar sap.** The previous model ('mustard oil bomb', Luthy & Matile, 1984), documented by fractionation studies, provided good evidence for the subcellular compartmentalization of myrosinase; i.e. that myrosinase was a cytosolic enzyme which was localized on the cytoplasmic face of the tonoplast and that glucosinolates were inside the vacuole. **Our results, employing immunogold localization, showed that the enzyme myroainase can be localized in the vacuoles in root cap and root cortex cells of the root tip in older tissues, thus myrosin grains developing inside myrosin cells (idioplasts) in very young tissues i.e. cotyledons and radicles are not the only location of the enzyme.** Previous workers did not detect myrosin cells in older roots than 48h , although they detected myrosinase activity; Iversen (1979) working with *S.alba* detected myrosin cells in the radicle after 2h, in the tap root after 24h, and in the root tip after 48h of imbibition respectively. In older tissues he did not detect myrosin (typical) cells but myrosinase activity was still detected. The some conclusion was reached by Phelan & Vaughan (1980) who detected myrosinase activity in organs that lacked myrosin cells. Additionally, Bones & Iversen (1985) detected myrosin cells in hypocotyls of seven different species of Brassicaceae (including *B.napus*) , up to 48h imbibition, in cotyledons up to 192h imbibition, and in roots up to 48h ,using different stains and the light microscope, although myrosinase activity was detected in older tissues. Iversen (1970a, 1973), using histocytochemical techniques, localized the myrosinase activity in membranes of mitochondria, dilated cisternae of ER and the nuclear membrane in the root cap, the zone of elongation and the root hair zone, of roots of 5 day old-seedlings of *Sinapis alba.*

The results presented in this study, help to explain the contradiction of the work of previous workers working with Brassica roots older than 48h after imbibition, who detected myrosinase activity in the absence of distinct cells viz. myrosin cells. The enzyme is in fact localized in in very young tissues i.e. radicles and cotyledons in myrosin grains in specific idioplasts (myrosin cells). In older roots, the enzyme is found in specific bodies (mab), inside the vacuoles, in the root tip (root cap and outer root cortex) of the developing plant. Thus, myrosinase activity can be detected in older roots, even though at this later stage specific idioplasts, myrosin cells (typical cells with myrosinase-accumulated grains) can not be detected. In all tissues, the enzyme is localized inside the vacuole.

In the ultrastructural studies in the present work, fungal hyphae were not detected in any of a number of sections that were made from root tips of plants that had been inoculated with either the virulent isolate (161) of the pathogenic diploid strain *V.d.longisporum*, or the avirulent isolate (130) of the haploid strain of *V.dahliae*. The only evidence of the fungus was a hypha of isolate 130 found in a cell of the root

cortex (oilseed rape cv. Cobra) that appeared to be undergoing an hypersensitive reaction (HR). This was obtained by Miss S. Garvin (Wye College, London) from a conventional stained section of root tip material that had been provided by the present author.

In a final attempt to localize the enzyme *in situ* in relation to the fungal propagules with the host-plant, stems of plants (oilseed rape cv. Cobra) that had been inoculated by the root-dipping method of inoculation with either the virulent isolate (161) or the avirulent isolate (130) were prepared for EM immunogold-labelling studies (2.5.2.2.1). In control plants, the enzyme was localized in cells that were occupied by a 1-3 large vacuoles which showed a positive reaction to the myrosinase antibodies. These cells were located in the phloem, and the enzyme was apparently restricted to these particular cells. Again here, the enzyme was localized in the vacuoles of particular cells in the phloem. The enzyme was not found in any other organelle, being restricted to vacuoles of these specific cells in the phloem. Inside the vacuole it was distributed uniformly and was always associated with e-opaque areas inside the vacuole sap. Xylem parenchyma cells of stems of control, and '130-inoculated' (incompatible interaction) plants lacked any labelling. On the contrary, in infected tissue (stems of '161-inoculated plants', compatible interaction), xylem parenchyma cells were disrupted and some of these showed cellular necrosis. The marked difference was that contact xylem cells (normally living cells that locate close to xylem vessels) showed a positive enzyme labelling in their vacuoles. From a number of studies involving *Verticillium* species and *Fusarium oxysporum*, these contact parenchyma cells have been shown to react to fungal invasion and to show differential responses in incompatible and compatible interactions (Muller & Beckman, 1984; Moreau & Catesson 1985; Muller & Beckman 1988; Beckman *et al.*, 1989; Tessier *et al.*, 1990; Beckman *et al.*, 1991; Mueller & Morgan 1993). A number of further experiments were performed in the present study, but the localization of the avirulent isolate 130 was very difficult to determine. For this purpose, preliminary experiments were conducted with a cut-stem inoculation technique (2.5.2.2.1), introducing the fungal propagules (spores) directly in the xylem. This was the only method that allowed as to observe the 130 avirulent isolate in the xylem vessels. However, the short time (90 min) of incubation with the fungus in the xylem was not sufficient to reveal any differences among the treatments. However the enzyme myrosinase was again localized in the same phloem specific cells as described above, for all treatments.

The induction of myrosinase activitythat was observed in later stages (15 and 20 days post-inoculation) of the development of the disease in the compatible (161) interaction could represent a late response of the host plant and is probably the same reaction that occurs in earlier stages in the incompatible (130) interaction. The increased levels of gluconasturtiin (GLNS), gluconapin (GLNP) and myrosinase activity detected here in earlier stages in the incompatible interaction, along with the delayed increases in levels of GLNS , GLNP and the late increase of the enzyme myrosinase *in situ* in the compatible (161) interaction, all support the hypothesis that this system is part of the complicated arsenal of plants, that is activated in different time periods and which nevertheless allow the virulent (compatible) isolates enough time to colonize the host plant before operation of defence mechanisms becomes critical for the pathogen. The number of experiments that were performed with control sections during this present study localized the enzyme in strategically key sites (cells in the outermost cell layer of the radicles, in the root cap and the first layer underneath the epidermis of the root tip) which also supports the previous

statement. Clearly, this is a fruitful area of research and more comparative studies are needed with different cultivars and different isolates of the haploid strains of *V.dahliae*, or isolates of the diploid strain *V.d.longisporum*, using cut-stem inoculations, to attempt to observe **early responses of the immediate cells close to fungal propagules of either virulent or avirulent isolates.** It would be very interesting in this context to study differences in compatible and incompatible interactions involving the parent isolate G22 of the diploid strain *V.d. longisporum* and its haploid recombinant detected in the present study,. that was shown subsequently to be avirulent to oilseed rape.

Symptoms in oilseed rape under semi-controlled, greenhouse conditions, were not of true wilt involving loss of turgor of leaves, but rather the leaves at first developed chlorosis, and then became necrotic and progressively desiccated. In an attempt to elucidate the possible symptom development, stems and petioles of oilseed rape plants (cv. Cobra) infected with the virulent isolate (161) of the diploid pathogenic strain *V.d.longisporum* were prepared for EM conventional studies. Ultrastructural studies of the vascular colonization by *V.d.longisporum* (isolate 161) revealed the same features of vascular colonization that had been previously observed in ultrastructural studies in other wilt diseases (Robb *et al.*, 1979a,1979b; Beckman *et al.*, 1982; Bishop & Cooper, 1983a, 1983b; Street *et al.*, 1986; Newcombe & Robb, 1988). The fungus caused extensive degradation of both primary wall of pit membranes and erosion of xylem vessel secondary walls, the latter requiring presumably the action of both pectic enzymes (PME, PL, PG) (Beckman *et al.*, 1987) and possibly lignin degrading enzymes (although apparently there is no information about the latter enzyme in vascular wilt diseases as far as it is known). In addition, in the present study, extensive wall degradation was observed in the absence (locally) of the fungus that means that the fungus secretes such CWDE (cell wall degrading enzymes) into the vascular fluid ahead of its own growth/colonization. The infection was accompanied by death of adjacent xylem parenchyma cells that could be a result of either : (a) direct (release of toxins/enzymes by the fungus) or (b) indirect (result of water stress, or by the toxic products of the glucosinolate break-down products) causes. Some of the xylem contact parenchyma cells that accompanied vessels that lacked the fungus were observed to be undergoing an active metabolic phase that could be prior to a hypersensitive (HR) cell death and might be associated with phytoalexin synthesis (Davys *et al.*, 1988; Rouxel *et al.*, 1989, 1990, 1991). The xylem vessels of infected stems and petioles were occluded by fungal spores and hyphae, tyloses and 'coating materials'. Three types of 'coating materials' observed have all been described previously by Robb *et al.* (1979a, 1979) and Bishop & Cooper (1983b). 'Smooth' and 'bubbly' coating materials lining the xylem vessel secondary walls and pit membranes, as well as a fibrillar material, were found most frequently and also coated the fungal propagules. This fibrillar coating on the fungus was connected with the fibrillar material on the vessel walls. These vascular coatings that form on xylem vessel walls are known to be common features in many other plants following various biotic and abiotic stresses (Robb *et al.*, 1980) and have been shown by the latter author to be important as defence mechanism of the host plant in vascular wilt diseases involving *Verticillium* species.

Conclusion

To conclude, the results of this study taken as a whole, indicate that:

(1) Verticillium wilt of oilseed rape [and other members of the Brassicaceae (Cruciferae)] is caused by the 'diploid' (probably a partial diploid) strain, formed as a result of inter-specific hybridization between two unknown haploid strains of *V.dahliae* and *V.albo-atrum*, which has produced the host-adapted pathogen *V.dahiae* var. *longisporum* Stark (1961), now proposed here as *V.cruciferarum* sp. novo Karapapa *et al.* (1994).

(2) This pathogen appears to overcome the myrosinase/glucosinolate non-specific, host defence mechanism in oilseed rape (and probably other Brassica/Crucifer plants) by suppression/avoidance of both myrosinase activation and the early synthesis of gluconasturtiin (GLNS) and gluconapin (GLNP) [as well as possesing relative tolerance/insensitivity to the myrosinase catalysed breakdown products of these specific glucosinolates, as demonstrated earlier by Baig (1991)].

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